

Glucose Metabolism in CD4⁺ T cell Subsets Modulates Inflammation and Autoimmunity

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Understanding the mechanisms that control T cell function and differentiation is crucial to develop new strategies to modulate immune function and prevent autoimmune and inflammatory disease. The balance between effector (Teff; Th1, Th2 and Th17) and regulatory (Treg) T cells is critical to provide an appropriate, but not excessive, immune response and therapies to induce Treg or inhibit Teff are likely promising treatment strategies. It has recently become clear that T cell metabolism is important in both T cell activation and differentiation. T cells undergo a metabolic reprogramming upon activation and not all differentiated T cell subsets utilize the same metabolic fuels or programs.

These metabolic differences are not trivial, as T cell metabolism is tightly regulated and dysregulation can lead to cell death or reduced immunity. An understanding of the metabolic differences between Teff and Treg may lead to a new direction for treating inflammatory diseases by modulating the Teff:Treg balance through metabolic inhibition. Previous studies have shown that Teff express higher levels of the glucose transporter Glut1 than Treg, however the role of Glut1, and importantly, the cell-intrinsic role of glucose metabolism in T cell differentiation and inflammation was not previously examined. The work presented here examines the role of Glut1 in T cell differentiation. We show that effector CD4 T cells were dependent on

Glut1 for proliferation and function both *in vitro* and *in vivo*. In contrast, Treg were Glut1-independent and capable of suppressing colitis in the absence of Glut1 expression.

Additionally, previous studies have shown broad metabolic differences between Teff and Treg, however the specific metabolic profiles of Teff and Treg are poorly understood. Here, Teff and Treg metabolism is examined to test if dependence on distinct metabolic pathways will allow selective targeting of different T cell populations. We show that pyruvate dehydrogenase kinase 1 (PDHK1) is differentially expressed in the T cell subsets and inhibition of PDHK1 selectively suppresses Th17 and promotes Treg differentiation and function. Because Teff and Treg have distinct metabolic profiles, we hypothesized that the Treg-specific transcription factor FoxP3 may drive the Treg oxidative metabolic program. We therefore examined the role of FoxP3 in T cell metabolism and determined that FoxP3 promotes glucose and lipid oxidation and suppresses glycolytic metabolism. Importantly, we show that promoting glycolysis with transgenic expression of Glut1 inhibits Treg suppressive capacity. Together, this data suggests that FoxP3 drives an oxidative metabolic program that is critical to Treg function. Overall, this work examines the metabolic phenotypes and regulation of Teff and Treg and potential metabolic targets that could be used to treat autoimmune and inflammatory disease.

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List of Abbreviations

2DG – 2-deoxyglucose

AMPK – AMP-activated protein kinase

APC – antigen presenting cell

ATP – adenosine triphosphate

CamK – Ca^{2+} /calmodulin-dependent kinase

cAMP – cyclic adenosine monophosphate

ConA – concanavalin A

CPT1 α – carnitine palmitoyltransferase 1 alpha

CTLA-4 – cytotoxic T-lymphocyte antigen 4

DCA – dichloroacetate

DLAT – dihydrolipoamide S-acetyltransferase

DLD – dihydrolipoamide dehydrogenase

DN – double negative

DP – double positive

EAA – essential amino acids

EAE – experimental autoimmune encephalomyelitis

ERR α – estrogen related receptor alpha

ETC – electron transport chain

FAD – flavin adenine dinucleotide

FoxP3 – forkhead box P3

G6P – glucose-6-phosphate

GATA-3 – GATA binding protein 3

Glut – glucose transporter

GSH – glutathione

GTP – guanosine triphosphate

GvHD – graft-vs-host disease

HIF α – hypoxia-inducible factor 1 alpha

Hk – hexokinase

IBD – inflammatory bowel disease

IFN γ – interferon gamma

IPEX – immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

iTreg – inducible regulatory T cell

LDH – lactate dehydrogenase

MAPK – mitogen-activated protein kinase

MHC – major histocompatibility complex

mTor – mammalian target of rapamycin

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

NFAT – nuclear factor of activated T cells

nTreg – natural regulatory T cell

OAA – oxaloacetate

OXPHOS – oxidative phosphorylation

PDH – pyruvate dehydrogenase complex

PDHK – pyruvate dehydrogenase kinase

PDP – pyruvate dehydrogenase phosphatase

PFK1 – phosphofructokinase 1

PI3K – phosphatidyl-inositol-3-kinase

PPP – pentose phosphate pathway

PTEN – phosphatase and tensin homolog

Rheb – Ras homolog enriched in brain

ROR γ t – RAR-related orphan receptor gamma

SCFA – short chain fatty acids

SGLT – sodium-glucose linked cotransporter

SLE – systemic lupus erythematosus

SP – single positive

SRC – spare respiratory capacity

SREBP1 – sterol regulatory element-binding protein 1

Stat – signal transducer and activator of transcription

T-bet – T-box expressed in T cells

TCA – tricarboxylic acid cycle

TCR – T cell receptor

Teff – effector T cell

TGF β – transforming growth factor beta

TPP – thiamine pyrophosphate

TRAF6 – TNF receptor-associated factor 6

Treg – regulatory T cell

TSC2 – tuberous sclerosis 2

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1. Introduction

CD4 T cells are a major component of the adaptive immune system. They direct the immune response and provide protection against foreign pathogens. Upon activation, CD4 T cells can differentiate into a variety of effector cells, including Th1, Th2 and Th17. These effector T cells coordinate the immune system depending on the type of response needed for a given pathogen. This involves diverse functions including activating B cells, recruiting neutrophils and eosinophils, and directing macrophage differentiation (reviewed in Zhu *et al.*, 2010). CD4 T cells can also differentiate into regulatory T cells, which are important in immune tolerance and protection against autoimmunity. Together, effector and regulatory T cells balance each other to ensure an appropriate but not excessive immune response.

Critical to the immune response is the ability of T cells to rapidly proliferate upon T cell receptor (TCR) engagement and subsequent T cell activation. As each T cell is specific for any given antigen, rapid proliferation is necessary to mount an appropriate immune response (Moon *et al.*, 2007). T cell activation creates a significant metabolic demand, as a T cell needs to generate sufficient energy and biosynthetic precursors to clonally expand. Among these is a metabolic reprogramming from an energy-efficient oxidative metabolic state to a highly glycolytic metabolism likely to support increased protein and lipid synthesis, cell growth and effector functions (Frauwirth *et al.*, 2004; Fox *et al.*, 2005).

It is now becoming apparent that T cell metabolism can have an enormous impact on T cell activation and differentiation (reviewed in MacIver *et al.*, 2013). Specific metabolic pathways support particular cellular functions and therefore naïve, effector, regulatory and memory T cells have distinct metabolic requirements. Importantly, these metabolic differences may provide an opportunity to modulate the balance between the CD4 subsets, particularly effector and regulatory T cells. Therefore, targeting CD4 T cell metabolism may provide new directions to modulate the immune response and treat autoimmune or inflammatory conditions.

1.1 CD4 T cell development

CD4 T cells are a type of lymphocytes that play a critical role in the adaptive immune system. CD4 T cells develop in the thymus and are pluripotent, having the ability to differentiate into distinct subtypes depending on the cytokine environment during T cell activation. Depending on the type of immune response needed, CD4 T cells can then go on to activate B cells, cytotoxic CD8 T cells, macrophages or other cell types and direct the immune response appropriately.

T cell development is initiated when lymphoid progenitor cells arise in the bone marrow and then migrate to the thymus, an organ in the chest cavity located near the heart. At this point, the precursor T cells lack expression of both CD4 and CD8 co-receptors and are known as double-negative (DN) T cells. During the four DN stages of

T cell development, each identified by the expression of specific surface activation markers such as CD44 and CD25, $\alpha\beta$ -T cells rearrange the genes encoding the T cell receptor (TCR), eventually ending up with a mature $\alpha\beta$ antigen receptor (Godfrey *et al.*, 1993). The transition from DN3 to DN4, in particular, involves rapid cell proliferation as the selected cells expand in number (Shortman *et al.*, 1990). A subset of thymocytes also goes on to become natural regulatory T cells (nTreg, described below). After the DN stage, TCR-expressing thymocytes begin to express both CD4 and CD8 co-receptors, becoming double-positive (DP) thymocytes.

DP thymocytes continue to rearrange the TCR and those that can interact with the major histocompatibility complex (MHC)-antigen structures expressed by thymic epithelial cells receive signals required to maintain their viability. Those thymocytes with TCR that cannot interact with the MHC:peptide complexes eventually die of neglect (Robey *et al.*, 1994). This process is known as positive selection, and is necessary to generate T cells that will be able to recognize MHC antigen presentation. However, some of the T cells that survive positive selection are able to bind to self-antigen-MHC complexes; these cells have the capacity to cause autoimmune disease in the periphery. Therefore, a second process known as negative selection occurs. The thymocytes that bind strongly to self-antigen-MHC complexes receive signals that promote their apoptosis. At this stage, about 3% of all DP thymocytes are selected to become single positive (SP) cells, expressing either CD4 or CD8 alone (Germain, 2002). At this point,

there is a second wave of thymocyte proliferation to expand the SP thymocyte numbers (Shortman *et al.*, 1990). SP cells then exit the thymus and enter the periphery as mature naïve T cells, waiting for the proper signals to activate and differentiate.

1.2 CD4 T cell activation

After a naïve CD4 T cell enters the periphery, it may encounter an MHC class II antigen presenting cell (APC) presenting antigen that is recognized by its TCR. If this binding occurs along with a secondary co-stimulatory signal, such as CD28 binding to B7.1 or B7.2 on the APC, the T cell will then undergo rapid expansion and activation (June *et al.*, 1987). T cell activation is accompanied by changes in gene expression, signaling pathways, proliferation, cytoskeleton rearrangements and metabolism. A series of tyrosine kinases, adaptor proteins and small GTPases direct the intracellular signaling pathways that initiate early T cell activation (Koretzky *et al.*, 2003). Along with other changes, calcium release from the endoplasmic reticulum activates calcium/calmodulin-dependent kinases (CamK) and calcineurin, which in turn activates mitogen-activating kinases (MAPK) and nuclear factor of activated transcription factors (NFAT), among others (reviewed in Smith-Garvin *et al.*, 2009).

Additionally, an important aspect of T cell stimulation is the co-stimulation of other cell surface receptors. Indeed, activation of the TCR without co-stimulation leads to T cell anergy, in which T cells are functionally inactivated (Schwartz, 2003). One of

these co-stimulatory receptors is the molecule CD28. CD28 activates the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, thereby promoting cell growth, metabolic changes and cytokine production (Lenschow *et al.*, 1996). Together, these and additional signaling pathways lead to the increased cell growth, proliferation, cytokine production and cytoskeletal changes that are characteristic of T cell activation.

1.3 CD4 T cell differentiation

Depending on the cytokine environment at the time of T cell activation, a CD4 T cell can differentiate into a number of fates, including but not limited to Th1, Th2, Th17 and Treg (**Fig 1.1**). Th1, Th2 and Th17 are known as effector T cells (Teff). These subtypes each play a unique role in the immune system, coordinating the immune response depending on the immune threat at hand. Regulatory T cells (Treg) instead function to actively suppress Teff proliferation to prevent excessive inflammation or autoimmunity. Therefore, a balance between each of these subtypes is important to provide appropriate immune protection without autoimmunity or immune suppression.

1.3.1 Th1 cells

Th1 cells were one of the first T cell subsets to be described and they function to facilitate the immune response against intracellular pathogens by activating macrophages and other phagocytes. Th1 cells were initially characterized as a type of

immune cell that produce the cytokines IL-2 and interferon gamma (IFN γ) upon activation (Mosmann *et al.*, 1986). Several years later, Hsieh *et al.* reported that Th1 cells could be generated *in vitro* by culturing naïve T cells with IL-12 produced by Listeria-induced macrophages (Hsieh *et al.*, 1993). It was later found that IL-12 functions by activating the signal transducer and activator of transcription 4 (Stat4) signaling pathway, which leads to the expression of the Th1-specific transcription factor T-box transcription factor TBX21 (T-bet) (Szabo *et al.*, 2000).

T-bet expression plays a central role in the differentiation of Th1 cells, activating the expression of IFN γ and actively repressing the Th2 differentiation pathway (Hwang *et al.*, 2005). Confirming the importance of this signaling pathway to Th1 differentiation, mice lacking either IL-12, Stat4 or T-bet cannot mount a Th1-driven immune response (reviewed in Gately *et al.*, 1998). Additionally, T-bet knockout mice spontaneously develop an asthma disorder, likely because of an abundance of Th2 in the absence of Th1 cells (Finotto *et al.*, 2002). In addition to T-bet, the cytokine IL-2 was found to be critical to Th1 differentiation and the induction of IFN γ (Liao *et al.*, 2011). Th1 cells are also involved in autoimmune disease and have been implicated in the pathogenesis of inflammatory bowel disease (IBD), arthritis and type 1 diabetes, among others (Lazarevic *et al.*, 2011).

1.3.2 Th2 cells

Along with the discovery of Th1 cells, Mosmann and Coffman also described a second population of T cells, Th2 cells. Whereas Th1 cells produce the cytokines IL-2 and IFN γ , Th2 were found to produce IL-4, IL-5 and IL-13 (Mosmann *et al.*, 1989). Th2 cells were also found to play a distinct role from Th1 in the immune response, being responsible for antibody responses and immunity to extracellular pathogens. The cytokines IL-4 and IL-5, in particular, activate and recruit B cells and eosinophils to drive the extracellular immune response. Naïve T cells differentiate into Th2 cells in the presence of TCR stimulation and the cytokine IL-4 (Le Gros *et al.*, 1990). IL-4 functions to activate the Stat6 signaling pathway and subsequently the transcription factor GATA-binding protein 3 (GATA-3) (Zheng *et al.*, 1997).

GATA-3 was originally described as a transcription factor involved with T cell development and is required for the transition from DP to SP thymocytes (Pai *et al.*, 2003). However, GATA-3 was later found to be involved specifically with Th2 differentiation, both by activating the expression of IL-4 and suppressing the expression of the Th1 cytokine IFN γ . Indeed, T cell specific GATA-3 knockout mice show a decreased ability to generate Th2 cells, instead producing IFN γ even in the presence of IL-4 (Zhu *et al.*, 2004). In addition to their role in the immune system, Th2 are also involved with the induction of allergy and asthma responses mediated through their activation of B cells, mast cells and basophils (reviewed in Hammad *et al.*, 2006).

1.3.3 Th17 cells

After the discovery of Th1 and Th2 cells, immunologists spent the next thirty years with the notion that no other effector T cell subset existed. However, as a better understanding of immune pathology emerged, it was clear that the Th1/Th2 hypothesis needed to be revised. One of the first signs that another T cell subset existed was data generated in the mouse model experimental autoimmune encephalomyelitis (EAE). EAE is a mouse model of neuroinflammation and multiple sclerosis. EAE was proposed to be mediated by Th1 cells, and would therefore be predicted to worsen with increased IFN γ expression. However, treatment of mice with IFN γ protected from EAE, and instead IFN γ knockout mice had more severe EAE than wild type mice (Voorthuis *et al.*, 1990).

Data soon emerged that both IL-6 and IL-17 knockout mice showed resistance to EAE (Samoilova *et al.*, 1998; Komiyama *et al.*, 2006). This led to the discovery of Th17 cells as a new class of effector T cells. Th17 were later shown to be induced by TCR stimulation in the presence of both IL-6 and transforming growth factor beta (TGF β) (Veldhoen *et al.*, 2006; Bettelli *et al.*, 2006; Mangan *et al.*, 2006). Th17 cells produce the cytokine IL-17 as well as IL-22 and IL-23. IL-17 can induce the expression of other inflammatory cytokines as well as activate neutrophils. Similarly to Th2, Th17 cells are involved in the immune response against fungi and extracellular bacteria, but importantly are specifically involved in mucosal immunity. Interestingly, Th17 may be

important in the regulation of the resident gut microbiota and distinguish between “good” and “bad” bacteria (Weaver and Hatton, 2009). Th17 cells also contribute to many different autoimmune diseases, including those that were once thought caused exclusively by Th1 cells including IBD, EAE and psoriasis (Zheng *et al.*, 2007; reviewed in Steinman 2007).

RAR-related orphan receptor gamma ($\text{ROR}\gamma\text{t}$) was determined to be the master regulator of Th17 cells when $\text{ROR}\gamma\text{t}$ knockout mice were shown to have decreased IL-17 production and importantly were resistant to the induction of EAE (Ivanov *et al.*, 2006). The Stat3 signaling pathway was also found to be critical in Th17 cells, as deletion of Stat3 led to the loss of IL-17 and IL-23 producing cells (Harris *et al.*, 2007). When both $\text{TGF}\beta$ and IL-6 are present, Stat3 activation leads to Th17 differentiation and $\text{ROR}\gamma\text{t}$ expression.

1.3.4 Regulatory T cells

Regulatory T cells (Treg) play an important role in maintaining immune tolerance. They have the ability to suppress the activation or proliferation of other T cells, thereby preventing an excessive immune response or autoimmunity. Treg that develop naturally in the thymus together with normal T cell development are called natural Treg (nTreg) (Hsieh *et al.*, 2004). However, naïve peripheral T cells can also differentiate into “inducible” Treg (iTreg). Both nTreg and iTreg are characterized by

the expression of the surface marker CD25, the alpha chain of the IL-2 receptor, as well as the transcription factor Forkhead Box 3 (FoxP3) (Fontenot *et al.*, 2003; Hori *et al.*, 2003). Although activated T cells express a transient level of CD25, Treg have a higher expression level and it is expressed constitutively. The importance of Treg becomes clear in mice with a loss-of-function mutation in FoxP3; the mice develop a fatal autoimmune disease and die within a month (Brunkow *et al.*, 2001). Similarly, humans with mutations in the FoxP3 gene have a disorder called immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and many do not live past the age of two due to extensive multi-organ autoimmune disease and eventual organ failure (Bennett, *et al.* 2001).

Treg exert their suppressive function by both cell-to-cell contact and cytokine-mediated methods. Treg were shown to inhibit the TCR-induced proliferation and IL-2 expression of T_H1 during cell-to-cell contact (Thornton *et al.*, 1998). Treg also express the inhibitory surface marker Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) that may contribute to T_H1 suppression (Takahashi *et al.*, 2000). Treg secrete several cytokines including TGF β and IL-10. TGF β is also critical in the differentiation of naïve T cells into iTreg (Chen *et al.*, 2003). IL-10 production has been shown to be essential in the control of colitis, as IL-10 deficient Treg were unable to prevent colitis in a mouse model (Asseman *et al.*, 1999).

Recent literature has suggested that Treg may also suppress effector T cells by metabolic interference. Adenosine is a critical component of both adenosine triphosphate (ATP) and the signaling molecule cyclic adenosine monophosphate (cAMP). FoxP3 drives the expression of CD39, an enzyme that degrades ATP to AMP and suppresses Teff through activation of the adenosine receptor 2A (Deaglio *et al.*, 2007). A second group showed that Treg could transfer cAMP into Teff through gap junctions as an additional suppressive mechanism (Bopp *et al.*, 2007). Additionally, Treg expression of CTLA-4 triggers a signaling response in dendritic cells upon binding to CD80 that inhibits glutathione (GSH) synthesis. This decrease in GSH limits cysteine availability and inhibits Teff activation and proliferation (Yan *et al.*, 2010). Treg also directly compete with Teff for cysteine uptake *in vitro* (Yan *et al.*, 2010).

Treg also have distinct intracellular signaling events from Teff. In particular, while all T cells respond to the cytokine IL-2, it has a unique signaling pathway in Treg. In effector T cells, IL-2 binds to the high-affinity IL-2 receptor and activates the JAK/STAT, MAPK and PI3K/Akt/mammalian target of rapamycin (mTor) pathways during T cell activation. However, the PI3K/Akt/mTOR pathway is not activated in Treg (Bensinger *et al.*, 2004; Zeiser *et al.*, 2008). This is in part due to negative regulation of the PI3K pathway through phosphatase and tensin homolog deleted on chromosome 10 (PTEN). While PTEN is downregulated upon Teff activation, PTEN expression remains high in Treg. The suppression of PI3K/Akt/mTOR signaling is also important clinically,

as the mTOR inhibitor rapamycin was found to expand Treg *in vitro* and is used to prevent graft-vs-host disease (GvHD) in human patients (Battaglia *et al.*, 2006; Blazar *et al.*, 1997).

1.4 Memory T cells

Following pathogen clearance, the majority of T cells undergo apoptosis while a small percentage remain as memory T cells. These persisting antigen-specific T cells are maintained indefinitely and have the ability to rapidly respond to subsequent pathogen challenge (Badovinac *et al.*, 2002). The memory T cell pool remains relatively constant in size and is maintained by IL-7- and IL-15-driven homeostatic proliferation (Surh *et al.*, 2006). There are both CD4 and CD8 memory T cells, although memory CD8 T cells have been studied more extensively. Memory CD8 T cells are typically divided into two categories based on the expression of specific homing receptors and surface markers. Central memory T cells are defined as CD62L⁺CCR7⁺ and they localize to lymphoid tissues while effector memory T cells are CD62L⁻CCR7⁻ and persist in peripheral tissues (Woodland and Kohlmeier, 2009). Upon rechallenge, both types of memory T cells produce inflammatory cytokines including IFN γ and TNF while effector memory T cells also produce high levels of perforin and granzyme B to kill infected cells. Memory CD4 cells have been examined in the context of influenza A infection and found to promote the B cell and cytotoxic CD8 T cell responses in addition to having direct cytotoxic

effects (McKinstry *et al.*, 2012). Overall, memory T cells are an important part of the immune response upon re-exposure to a given pathogen.

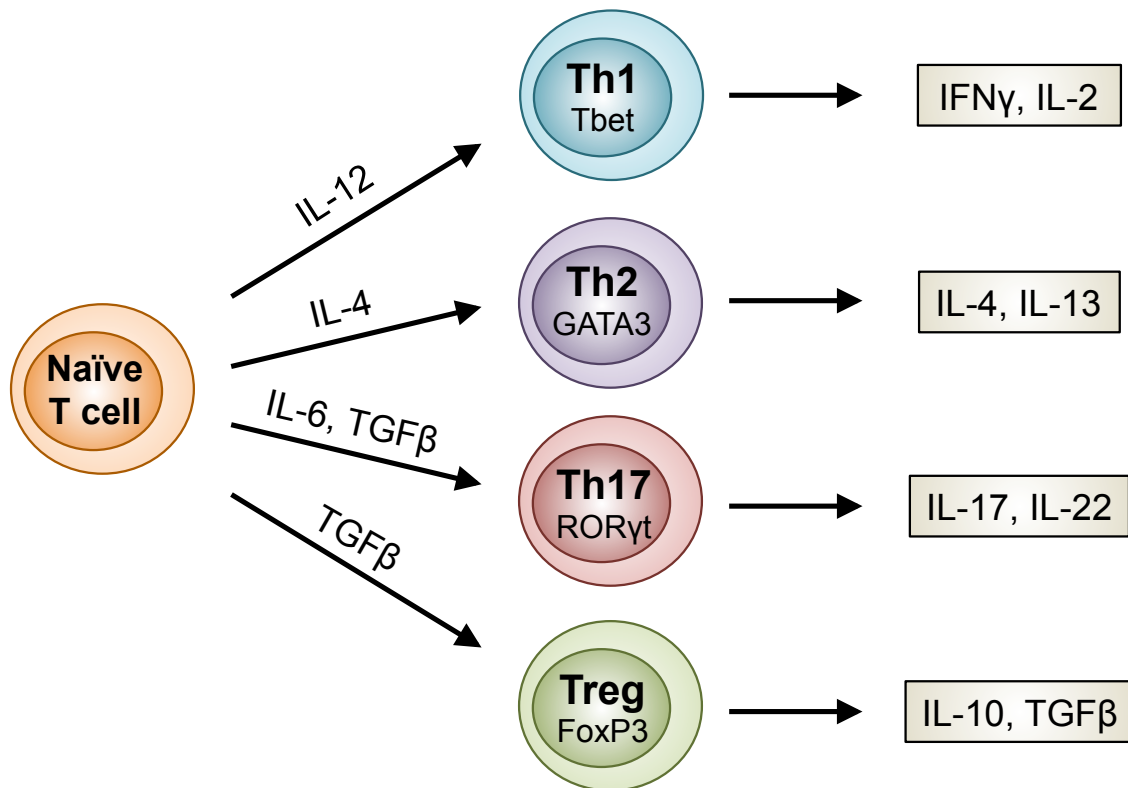


Figure 1.1: CD4 T cell subsets

Naïve T cells are pluripotent and have the ability to differentiate into Teff (Th1, Th2 and Th17) or Treg depending on the cytokine environment at the time of TCR stimulation. Each T cell subset has a unique transcription factor (Th1, T-bet; Th2, GATA-3; Th17, ROR γ t; Treg, FoxP3) and produces a unique set of cytokines.

1.5 Glucose Metabolism

Many different types of organisms use glucose as a main source of fuel. Glucose is particularly important in the mammalian brain, where it is used as the primary energy source during normal conditions. For this reason, if blood glucose levels drop too low, it is a dangerous and potentially fatal condition if not immediately corrected (Cryer *et al.*, 2007). At the same time, excessive glucose levels such as those that occur in the context of diabetes can result in peripheral vascular disease, renal failure and blindness (reviewed in Kaiser *et al.*, 2003). The liver plays a critical role in whole body glucose homeostasis and is one of the main glycogen stores (McGarry *et al.*, 1987). The regulation of plasma glucose levels is in part driven by changes to the levels of the hormones insulin and glucagon, which influence glucose metabolism in the liver and skeletal muscle. The tight regulation of glucose homeostasis is critical to maintain glucose levels at the appropriate level for normal bodily functions.

While glucose is important to an organism as a whole, glucose metabolism on a cellular level may be equally as important. T cells, in particular, require large amounts of glucose upon activation to provide energy for rapid growth and proliferation (Fox *et al.*, 2005). Glucose needs to first be imported into the cell where it can then be used to generate ATP energy or for other purposes such as protein glycosylation, conversion into lipids for building membranes, or for nucleotide biosynthesis. Cellular control of glucose metabolism occurs at both the level of glucose import, as well as the regulation

of glycolytic enzymes through signaling pathways that direct glucose towards different fates.

1.5.1 Glucose Transport: Glut1

For glucose to be metabolized intercellularly, it first needs to be taken up into the cell (**Fig 1.2, 1**). As the lipid membrane is not permeable to glucose, it is transported across the membrane by carrier proteins called glucose transporters. There are both sodium-coupled and sodium-independent glucose transport mechanisms to bring glucose into the cell. The sodium-coupled transporters comprise a family of sodium dependent glucose transporters (SGLT) and are primarily located in the intestines and renal system (Wright *et al.*, 2001). The SGLTs co-transport sodium and glucose, and contribute to the reabsorption of glucose in the renal system. The sodium-independent carriers are members of the facilitative glucose transporter (Glut) family. There are fourteen Glut transporters, which are expressed in a tissue-specific manner. All of the Gluts are membrane proteins and share a series of 12 transmembrane helices that are typically heavily glycosylated (Mueckler *et al.*, 1985). With the exception of Glut5, which transports fructose, the Glut transporters regulate glucose uptake and all have different binding affinities and K_m values that allows for specific glucose regulation needs depending on the cell type or organ system (Douard *et al.*, 2008).

Glut1 (gene name: SLC2A1) is the most abundantly expressed Glut transporter isoform and plays an important role in many tissues, particularly the brain where it transports glucose across the blood-brain barrier (Brockmann *et al.*, 2009). Glut1 is also expressed in activated lymphocytes, increasing drastically in expression level upon stimulation (Chakrabarti *et al.*, 1994). In T cells, it is highly regulated; cytokine stimulation promotes glucose uptake through Glut1 trafficking. In a related cytokine-dependent hematopoietic cell line, cytokine withdrawal led to Glut1 internalization and upon cytokine readdition, Glut1 was returned to the surface (Wieman *et al.*, 2007). This regulation occurs through the PI3K pathway, which promotes Glut1 trafficking to the cell surface (Wieman *et al.*, 2007; Bentley *et al.*, 2003). Inhibition of PI3K activity reduced surface Glut1 levels, while constitutive expression of active Akt was sufficient to maintain surface Glut1 levels even in the context of cytokine withdrawal.

1.5.2 Aerobic Glycolysis

After glucose gets transported into the cell, it has three major metabolic fates. Glucose can be fully oxidized to carbon dioxide and water, yielding ATP energy for the cell (**Fig 1.2, 4**). Glucose can also be shunted to the pentose phosphate pathway (PPP). The PPP generates both nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate, contributing to lipid synthesis and nucleotide synthesis, respectively (Hothersall *et al.*, 1979). Additionally, glucose can be reductively

metabolized to alcohols or organic acids including ethanol and lactate through the process of fermentation (**Fig 1.2, 3**). Unlike glucose oxidation, fermentation does not require oxygen but it is much less efficient at ATP energy generation. Indeed, many anaerobic organisms obtain energy through this pathway when oxygen is lacking (Rolland *et al.*, 2002).

However, in 1924 Otto Warburg found that mammalian cancer cells produce high levels of lactate from glucose even in the presence of oxygen (Warburg *et al.*, 1956). This has been termed aerobic glycolysis, as glucose is converted to lactate even in the presence of sufficient oxygen levels for glucose oxidation through the carboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Shortly thereafter, other groups found that other non-transformed cells also utilize aerobic glycolysis, particularly those that are highly proliferative (Wang *et al.*, 1976). This includes proliferating fibroblasts and both human and mouse lymphocytes.

Once glucose enters the cell, it is immediately converted into glucose-6-phosphate (G6P) by the enzyme hexokinase (Hk). The phosphorylation of glucose to G6P effectively traps glucose in the cytoplasm of the cell, as G6P cannot be transported back out of the cell (Berg *et al.*, 2007). There are several isoforms of hexokinase (Hk1-4) with varying tissue specificity and affinities for glucose. Hk1-3 are 100kD and thought to be due to a gene duplication of a 50kD ancestral Hk (Bork *et al.*, 1993). Hk4 is a 50kD protein, which is also referred to as glucokinase. Hk1 is ubiquitously expressed, while

Hk2 is found primarily in skeletal muscle and adipose tissue, both insulin sensitive tissues (reviewed in Wilson *et al.*, 2003; Postic *et al.*, 1994). Wilson *et al.* propose that the specific response of Hk2 to G6P and inorganic phosphate (Pi) suggest that it plays a more anabolic role by providing G6P to PPP (Wilson *et al.*, 1995). Data suggest that Hk1 and 2 are associated with the mitochondria, both having a hydrophobic N-terminal mitochondrial signaling sequence (Sui and Wilson, 1997). In contrast, Hk3 does not contain this signaling sequence. After 24 hours of activation, T cells express Hk1, 2 and 3 with Hk2 being the predominant isoform (Wang *et al.*, 2011).

After glucose is converted to G6P by Hk, another enzyme called phosphofructokinase (PFK1) commits it to the glycolytic pathway. PFK1 is regulated allosterically through ATP and citrate inhibition. Glucose is then further metabolized by other glycolytic enzymes to the final product of pyruvate. Overall, glycolysis generates 2 ATP molecules per molecule of glucose as well as reducing agents in the form of nicotinamide adenine dinucleotide (NADH) (Berg *et al.*, 2007). Pyruvate can then either be transported across the mitochondrial membrane and converted to acetyl CoA or converted to lactate in the cytoplasm.

The conversion of pyruvate to lactate is performed by an enzyme called lactate dehydrogenase (LDH) and this consumes NADH and recycles NAD⁺. This recycling is important, as cytosolic NAD⁺ is limiting and it is important for the cell to maintain a specific NAD⁺/NADH ratio (reviewed in Lunt *et al.* 2011). Although aerobic glycolysis is

much less efficient at ATP generation than glucose oxidation, it may be that the glycolytic flux in rapidly proliferating cells exceeds the capacity of pyruvate dehydrogenase (PDH) and other enzymes in the glucose oxidation pathway (Curi *et al.*, 1988). Therefore, glucose would be converted to lactate and allow NAD⁺ regeneration.

Another function of aerobic glycolysis in proliferating cells may be to contribute to the generation of biosynthetic precursors to support cell growth. Cell growth and proliferation requires the doubling of all cellular contents, including nucleotides, proteins and lipids; all of which can be generated from glucose (Berg *et al.*, 2007). Indeed, Newsholme *et al.* has suggested that lymphocytes perform glycolysis at a high rate to allow for glycolytic intermediates to be shunted off towards biosynthetic pathways that allow for rapid cell growth and division (Newsholme *et al.*, 1985a). However, the reasons why highly proliferative cells utilize aerobic glycolysis rather than OXPHOS are still under active investigation.

1.5.3 Pyruvate Dehydrogenase Kinase

The pyruvate dehydrogenase complex (PDH) converts pyruvate to acetyl CoA in the committal step of glucose oxidation. PDH is comprised of three subunits, pyruvate dehydrogenase, dihydrolipoyl transacetylase (DLAT) and dihydrolipoyl dehydrogenase (DLD). Pyruvate dehydrogenase uses the cofactor thiamine pyrophosphate (TPP, vitamin B1) to decarboxylate pyruvate. DLAT and DLD subsequently add coenzyme A

and reduce NAD^+ to NADH (reviewed in Patel and Korotchkina, 2006). Because PDH sits at the branch point between the conversion of pyruvate to lactate or oxidation to carbon dioxide, it is highly regulated.

PDH is regulated allosterically by its end products acetyl CoA, NADH and ATP, all of which lead to its inhibition. Additionally, the activity of PDH can be acutely regulated by the phosphorylation and dephosphorylation of three specific sites on the alpha subunit: Ser-203, Ser-264 and Ser-271 (Korotchkina *et al.*, 2001). Phosphorylation of PDH is inhibitory to the complex while the active form is dephosphorylated. PDH is specifically phosphorylated by the pyruvate dehydrogenase kinase (PDHK) family of enzymes. There are four PDHK isoforms (PDHK1-4) that have varying specificity for the three PDH phosphorylation sites. Only PDHK1 is able to efficiently phosphorylate all three sites (Korotchkina *et al.*, 2001). Additionally, there are two specific pyruvate dehydrogenase phosphatases (PDP1 and PDP2) that dephosphorylate the same three phosphorylation sites (Teague *et al.*, 1982).

In addition to phospho-site specificity, the four PDHK isoforms also differ in tissue distribution. PDHK1 is found primarily in the heart with lower levels in the liver and spleen, while PDHK3 is highly expressed in the testis and also found in the lung, brain and spleen. PDHK4 is expressed in the skeletal muscle and heart while PDHK2 is fairly ubiquitously expressed (Bowker-Kinley *et al.*, 1998). PDHK1 itself is allosterically regulated; inhibited by ADP and pyruvate and activated by NADH and acetyl CoA

(Roche *et al.*, 2001). Overall, PDH is an important and highly regulated step linking glycolysis to both oxidative phosphorylation and fatty acid synthesis.

Recently, a role for PDHK has been described in the cancer field. Many cancer cells, like T cells, undergo a metabolic transition from an oxidative metabolism to using glycolysis as a primary energy source (Gatenby *et al.*, 2004; Vander Heiden *et al.*, 2009). A 150 dalton compound called dichloroacetate (DCA) was found to inhibit all four of the PDHK isoforms in a dose-dependent manner (Stacpoole, 1989). DCA has been shown to promote cancer cell apoptosis *in vitro* and decreased tumor growth in mouse models (Bonnet *et al.*, 2007; Sun *et al.*, 2010). DCA is currently in clinical trials for several different types of cancer, however, at this time there is limited efficacy data and this continues to be an active area of investigation (Sutendra *et al.*, 2013; Garon *et al.*, 2014). In humans, DCA appears to have no significant toxicity other than reversible peripheral neuropathy at high doses, although more studies are needed (Michelakis *et al.*, 2010; Dunbar *et al.*, 2013). Additionally, PDH activity has been linked to oncogene-induced senescence and Kaplon *et al.* showed that PDK1 depletion is toxic to melanoma cells (Kaplon *et al.*, 2013). These data suggest that targeting PDHK is potentially an exciting metabolic therapeutic strategy for a variety of diseases.

1.5.4 Glucose and Lipid Oxidation

After glucose is taken up into the cell, it can be ultimately oxidized to carbon dioxide and water or converted to lactate. Under standard oxygen conditions, glucose is typically converted to pyruvate, which is transported into the mitochondria for further breakdown in the TCA cycle (**Fig 1.2, 5**). Pyruvate is first converted to acetyl CoA by PDH, as described above. Acetyl CoA enters the TCA cycle and combines with the four-carbon oxaloacetate (OAA) into the organic acid citrate. The TCA cycle consists of nine reactions, which ultimately remove two carbons in the form of CO₂ and recycle OAA. The catabolism of acetyl CoA yields both carbon dioxide and electrons, which are donated to the electron carriers NAD⁺ and flavin adenine dinucleotide (FAD⁺) to make NADH and FADH₂, respectively. Additionally, a guanine triphosphate (GTP) is generated by substrate level phosphorylation. Overall, six CO₂ molecules, 8 NADH and 2 FADH₂ are generated per glucose in the TCA cycle (Lodish *et al.*, 2000).

ATP generation is coupled to the re-oxidation of NADH and FADH₂, which donate electrons to the electron transporter chain (ETC). This process requires oxygen as the final electron acceptor. The movement of electrons is coupled with the transfer of protons from the inner mitochondrial membrane to the intermembrane space, setting up an electrochemical gradient. This gradient is then utilized by F₀F₁ ATP synthase to generate ATP (Mitchell *et al.*, 1961). Protons pass through the F₀ subunit, following their concentration gradient and the energy released is used to convert ADP and inorganic

phosphate to ATP in the F1 subunit. Therefore, glucose is fully oxidized in the TCA cycle to CO₂ and the remaining electrons are put onto electron carriers and are donated to the electron transport chain to ultimately generate ATP energy for the cell.

Similarly to glucose oxidation, lipid oxidation also involves the generation of NADH and FADH₂ electron carriers as well as acetyl CoA molecules that enter the TCA cycle (**Fig 1.2, 6**). Long-chain fatty acids enter the mitochondria through a carrier system, the carnitine shuttle, while short-chain fatty acids can enter the mitochondria through diffusion. Even-numbered fatty acids are fully oxidized to acetyl CoA through a series of dehydrogenation and oxidization steps. Odd-numbered fatty acids yield acetyl CoA molecules plus propionyl CoA, which is ultimately converted to succinyl CoA (Lodish *et al.*, 2000) and enters the TCA cycle.

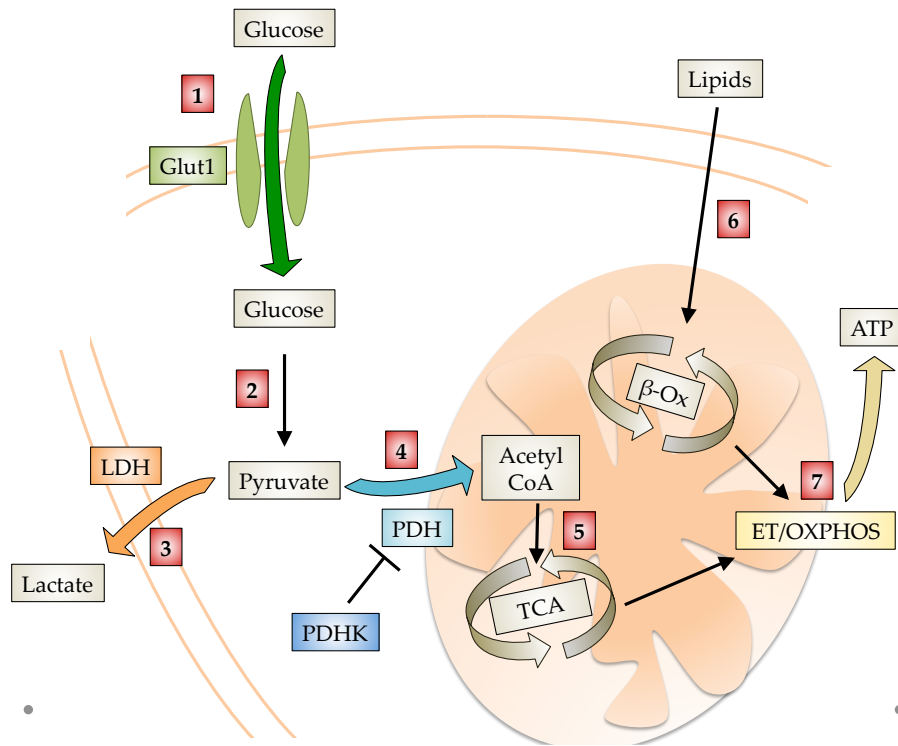


Figure 1.2: Metabolic pathways and fates of glucose and lipids

Glucose enters the cell via glucose transporters such as Glut1 (1). Glucose is then converted into pyruvate in the glycolytic pathway (2). Pyruvate can be converted to either lactate via lactate dehydrogenase (LDH, 3) or to acetyl CoA by pyruvate dehydrogenase (PDH, 4). PDH is in part regulated by a group of kinases called the pyruvate dehydrogenase kinases (PDHK). Acetyl CoA generated from either glucose or lipids is then oxidized in the carboxylic acid (TCA) cycle (5). In contrast to glucose, lipids enter the cell via diffusion through the lipid bilayer and undergo beta-oxidation (β -Ox, 6) where they are broken down to acetyl CoA and then are further oxidized in the TCA cycle similarly to glucose. The electrons removed during the TCA cycle are placed on electron carriers and shuttled to the electron transport chain (ET). The ET chain is coupled with oxidative phosphorylation (OXPHOS) and generates ATP energy (7).

1.6 Immunometabolism

T cells are critically important to provide protection against foreign pathogens. T cell activation leads to an extremely high rate of growth and proliferation, which is necessary to provide sufficient immune protection. To fuel this proliferative demand, T cell metabolism is reprogrammed to provide the necessary energy and biosynthetic precursors to support growth and effector function. Furthermore, naïve, effector, regulatory and memory T cells have distinct roles in the immune system and with those different functions comes unique metabolic requirements (**Fig 1.3**, reviewed in Gerriets *et al.*, 2012). Therefore, lymphocyte metabolism is intimately linked to immune function and an understanding of these metabolic differences and requirements may provide new opportunities to treat autoimmune and inflammatory diseases.

1.6.1 Naïve T cell metabolism

After positive and negative selection in the thymus, naïve T cells enter the periphery and migrate between the spleen, lymph nodes and peripheral lymphoid organs. Naïve T cells require only low levels of metabolic fuels to provide energy for basal functions, including migration, homeostatic proliferation and immunosurveillance. To perform these functions, naïve T cells utilize a variety of fuels including glucose, glutamine and fatty acids (Jones *et al.*, 2007). Importantly, they oxidize these fuels in the mitochondria and produce very little lactate (Ardawi *et al.*, 1984).

Naïve T cells rely on extrinsic signals, such as IL-7, to maintain their survival and metabolic phenotype (Rathmell *et al.*, 2001). Cells deprived of IL-7 are unable to maintain cell size or glucose uptake and will upregulate pro-apoptotic Bcl-2 family member proteins and undergo apoptosis (Coloff *et al.*, 2011; Rathmell *et al.*, 2000). The IL-7 receptor regulates glucose uptake in part through PI3K/Akt/mTor pathway regulation of Glut1 trafficking to the cell surface. This may allow naïve T cells to maintain survival via upregulation of glucose uptake and glycolysis (Wofford *et al.*, 2008). Indeed, conditional deletion of the IL-7 receptor in T cells leads to an inability to maintain glycolysis and leads to subsequent cell death (Jacobs *et al.*, 2010). This metabolic role of IL-7 is important to maintain homeostasis of T cell numbers without promoting autoimmune disease or immune suppression.

1.6.2 Metabolic reprogramming in T cell activation

After antigen presented by a stimulated antigen-presenting cell (APC) is recognized by a T cell along with appropriate co-stimulatory conditions, the T cell will become activated. Along with activation comes an enormous demand for rapid growth and proliferation to drive the immune response (Moon *et al.*, 2007). To fuel this demand, T cells have significantly increased metabolic requirements necessary to support cell growth and proliferation. T cell metabolism shifts from an oxidative, catabolic state to an anabolic state, as the proliferating T cell needs cellular building blocks including

protein, nucleic acids as well as lipids for building new membranes. This metabolic shift is reminiscent of many cancer cells, with a high rate of aerobic glycolysis and involves a drastic increase in glucose uptake and glycolysis as well as glutamine metabolism (Macintyre and Rathmell, 2013). In addition to metabolic reprogramming towards aerobic glycolysis, Chang *et al.* has also shown that oxidative phosphorylation is important for early T cell activation, as oligomycin treatment to inhibit ATP synthase blocked the expression of surface activation markers and prevented T cell proliferation following activation (Chang *et al.*, 2013).

The metabolic reprogramming that occurs upon T cell activation was originally described over thirty years ago in rat lymphocytes. Newsholme *et al.* showed that in concanavalin A (ConA) stimulated lymphocytes, carbon dioxide produced after the metabolism of glucose was far less than expected given the high rate of glucose uptake (Newsholme *et al.*, 1985b). Instead, lymphocytes only partially oxidize glucose and glutamine, instead converting glucose to lactate and glutamine to either glutamate or other amino acids. Glutamine was also found to be essential for lymphocyte proliferation, as replacement of glutamine with other amino acids did not rescue thymidine incorporation into DNA (Ardawi and Newsholme, 1983). Further work suggested that both glycolysis and glutaminolysis function at a high rate in order to allow the use of metabolic intermediates for biosynthesis of macromolecules (Newsholme *et al.*, 1985a). Importantly, these studies used ConA, a lectin that functions

as a mitogen, to activate lymphocytes and therefore it will be important to confirm these studies with physiological T cell stimulation.

These changes in metabolism are coordinated by various signaling events after T cell stimulation. Co-stimulation through CD28 activates the phosphatidyl-inositol-3-kinase (PI3K)/Akt/mTORC pathway to promote both Glut1 expression and trafficking to the surface (Jacobs *et al.*, 2008). The PI3K/Akt/mTORC pathway also activates lipid synthesis through sterol regulatory element-binding protein 1 (SREBP1) (Duvel *et al.*, 2010). In addition, c-Myc is important to promote genes associated with both glucose and glutamine metabolism early on in T cell activation (Wang *et al.*, 2011). Consequently, c-Myc knockout T cells cannot upregulate glycolytic and glutaminolytic genes and fail to activate. In addition to c-Myc, the nuclear hormone receptor estrogen-related receptor alpha (ERR α) has been shown to be important for T cell activation as well as differentiation. ERR α was initially described as a transcriptional regulator of mitochondrial biogenesis and oxidation that is important to regulate cell energy balance (Sladek *et al.*, 1997). ERR α expression is induced upon T cell activation and was shown to direct gene expression regulated to both glucose and mitochondrial metabolism, promoting T cell growth and proliferation (Michalek *et al.*, 2011b). T cells deficient in ERR α had decreased cytokine production, proliferation and glucose metabolism (Michalek *et al.*, 2011b). Together, these signaling pathways coordinate cell metabolism

to balance energy generation and biosynthesis for maximal T cell growth and proliferation rates.

While glucose and glutamine utilization increase upon activation, fatty acid oxidation is downregulated, likely because lipids are necessary to provide building blocks for new membranes. In hematopoietic cells, fatty acid oxidation is regulated in part through the PI3K/Akt/mTOR pathway, which can suppress the expression of carnitine palmitoyltransferase Ia (CPT1 α), the rate-limiting step of long-chain fatty acid oxidation (Deberardinis *et al.*, 2006). In addition, CPT1 α is likely allosterically inhibited by malonyl CoA, the end product of fatty acid synthesis, although this has not been shown in lymphocytes (McGarry *et al.*, 1977).

This switch in fuel usage during T cell activation is critical to support both growth and proliferation. Glucose starvation or limitation can inhibit T cell proliferation as well as cytokine production (Cham *et al.*, 2005; Jacobs *et al.*, 2008; Zheng *et al.* 2009). This occurs even in the presence of other fuel sources, suggesting that glucose is required for T cell activation and function (Jacobs *et al.*, 2008). Together, these data indicate that glucose and glutamine metabolism is a critical and necessary aspect of T cell activation.

1.6.3 Effector and Regulatory T cell metabolism

Along with TCR engagement and co-stimulation, the cytokine environment promotes CD4 T cells to undergo differentiation into effector T cells (Teff; Th1, Th2, Th17) or inducible regulatory T cells (Treg). Each of these subsets has a unique role to promote or suppress immunity. Along with unique immunological roles, recent data suggests that the CD4 T cell subsets may have specific metabolic requirements and programs. Metabolic differences between the subsets could potentially provide a way to regulate the immune response in the context of an autoimmune or inflammatory disorder.

Like activated T cells, effector T cells rely on glucose to support the bioenergetics needs of rapid growth and proliferation. Effector T cells express high levels of Glut1 and have high rates of glucose uptake and glycolysis (Michalek *et al.*, 2011a; Shi *et al.*, 2011). In contrast, Treg have lower rates of glucose uptake and glycolysis and a lower expression of Glut1, albeit higher than naïve T cells. *In vitro*, Teff are much more sensitive to 2-deoxyglucose (2DG) treatment to inhibit glycolysis, suggesting that there may be differences in glucose utilization between Teff and Treg (Shi *et al.*, 2011). Instead, we have found that Treg have a higher rate of lipid oxidation than Teff (Michalek *et al.*, 2011a). Furthermore, the addition of exogenous fatty acids promotes Treg formation but inhibits Teff. This suggests that Treg have more fuel flexibility and in the presence of glycolytic inhibitors, are able to rely on lipid oxidation.

The PI3K/Akt/mTOR pathway also plays an important metabolic role in the T cell subsets. This pathway both promotes Teff by upregulating glucose metabolism and inhibits Treg generation. T cell specific mTOR knockout cells fail to become effector T cells even when differentiated *in vitro* with the appropriate cytokines (Delgoffe *et al.*, 2009). Instead, the T cells become FoxP3⁺ Treg. In addition, the mTOR inhibitor rapamycin promotes the generation of Treg both *in vitro* and *in vivo* (Battaglia *et al.*, 2005), although the role of rapamycin is complex. There are two mTOR complexes, mTORC1 and mTORC2; mTORC1 is regulated by PI3K while mTORC2 promotes Akt and other related kinases (Chi, 2012). Interestingly, these two mTOR complexes have differential roles in effector T cell differentiation. Ras homolog enriched in brain (Rheb) knockout T cells lack mTORC1 activity and can differentiate into Th2 but not Th1 or Th17 (Delgoffe *et al.*, 2011). Alternatively, T cells lacking mTORC2 activity have the ability to become Th1 and Th17 but not Th2. Therefore, the mTOR signaling pathway has an important and complex role in T cell differentiation and it will be important to see how alterations in these pathways lead to changes in T cell metabolism.

In addition to the PI3K/Akt/mTOR signaling pathway, the opposing AMP-activated kinase (AMPK) may play a role in T cell differentiation. Treg have been shown to have a higher expression of phospho-AMPK than Teff (Michalek *et al.*, 2011b). AMPK senses metabolic stress and is allosterically activated by AMP and inhibited by ATP. When activated, AMPK increases catabolic processes including glucose uptake

and fatty acid oxidation, although this has not been shown in T cells (Mihaylova and Shaw, 2011). Treatment of mice with metformin, which leads to AMPK activation, can also induce Treg *in vivo* (Michalek *et al.*, 2011b). AMPK signaling opposes the mTOR pathway, as AMPK inhibits mTORC1 by the phosphorylation of tuberous sclerosis 2 protein (TSC2) and subsequent inhibition of Rheb (Chi, 2012). Therefore, the AMPK/mTOR signaling pathways appear to coordinate opposing metabolic programs in Teff and Treg.

In addition to the PI3K/Akt/mTOR signaling pathway, the transcription factor hypoxia inducible factor 1 alpha (HIF1 α) plays a role in T cell differentiation. Although HIF1 α has been shown to be dispensable in early T cell activation, it is selectively expressed in Th17 and important to upregulate glucose metabolism (Wang *et al.*, 2011; Shi *et al.*, 2011). This was shown to be dependent on mTOR signaling. HIF1 α also modulates the balance between Th17 and Treg, as a T cell specific HIF1 α knockout promotes Treg generation and inhibits IL-17 production *in vitro* and protects against EAE *in vivo* (Dang *et al.*, 2011). In this setting, HIF1 α acts to directly increase the activity of ROR γ t, the master regulator of Th17 and can also bind to FoxP3 and lead to its degradation (Dang *et al.*, 2011). Therefore, HIF1 α likely has both metabolic and non-metabolic roles to control T cell differentiation.

Complicating the understanding of Treg metabolism is recent data with the PDHK1 inhibitor DCA. As described above, DCA inhibits PDHK1, which in turn

activates PDH to drive glucose oxidation and subsequently reduce lactate production. Treatment of T cells with DCA promotes the generation of Treg (Ostroukhova *et al.*, 2012). *In vivo* DCA treatment inhibits airway inflammation and collagen-induced arthritis in mice (Bian *et al.*, 2009). Although these studies did not examine T cell metabolism in detail, this role of DCA suggests that glucose oxidation may be favorable for Treg. However, the role and requirement of mitochondrial oxidation of fuel for Treg differentiation or function is not yet understood.

1.6.4 Memory T cell metabolism

At the end of an immune response and pathogen clearance, TCR stimulation decreases and the majority of effector T cells die of apoptosis; however a few antigen-specific T cells persist as memory cells. Memory T cells are important to confer protection to pathogens that were previously seen by the immune system and upon secondary challenge with the same pathogen can give an enhanced and efficient response (reviewed in D'Cruz *et al.*, 2009). Memory cells therefore may require a unique metabolic program that allows for both long-lived quiescence as well as have the ability to rapidly respond to secondary infections. While memory CD4 T cell metabolism has not been extensively studied, recent studies have examined memory CD8 T cell metabolism.

The first evidence that memory T cells may utilize a unique metabolic program was when TNF receptor associated factor 6 (TRAF6) knockout T cells were shown to have defects in the transition between effector and memory phenotypes. TRAF6-deficient T cells were shown to have defects in fatty acid metabolism that could be rescued with metformin treatment (Pearce *et al.*, 2009). Subsequently, the same group showed that a cytokine important for T cell memory, IL-15, promotes the expression of CPT1 α and therefore lipid oxidation (van der Windt *et al.*, 2012). In addition, when CPT1 α was retrovirally expressed in T cells, it increased the recall response of CD8 T cells *in vivo*, suggesting that memory T cells require fatty acid oxidation to rapidly proliferate upon restimulation (van der Windt *et al.*, 2012; van der Windt *et al.*, 2013).

Similarly to Treg, CD8 memory T cells were also found to have high levels of AMPK activity, as measured by AMPK phosphorylation. Additionally, treatment of T cells with metformin *in vivo* promoted CD8 memory T cell accumulation (Pearce *et al.*, 2009). At the same time, Araki *et al.* showed that mTOR is an important regulatory of CD8 memory T cells, as rapamycin treatment to inhibit mTOR leads to increased memory formation and enhanced the effector to memory transition (Araki *et al.*, 2009). Therefore, promoting oxidative metabolism appears to be critical for memory T cell formation to allow recall responses.

In addition to the transition between glycolytic effector and quiescent memory cells, memory CD8 T cells need to be able to rapidly respond to secondary infection.

Van der Windt *et al.* showed that *in vitro* memory T cells have higher spare respiratory capacity (SRC), which is a measurement of the reserve capacity of the cell to increase oxygen consumption (Van der Windt *et al.*, 2012; Gubser *et al.*, 2013). Memory CD8 T cells were also shown to have more mitochondrial biomass than effector or naïve T cells. Together, this suggests that while memory T cells have a quiescent catabolic metabolism, they have the capacity to greatly increase their respiratory rate and energy production as needed for a rapid recall response to infection.

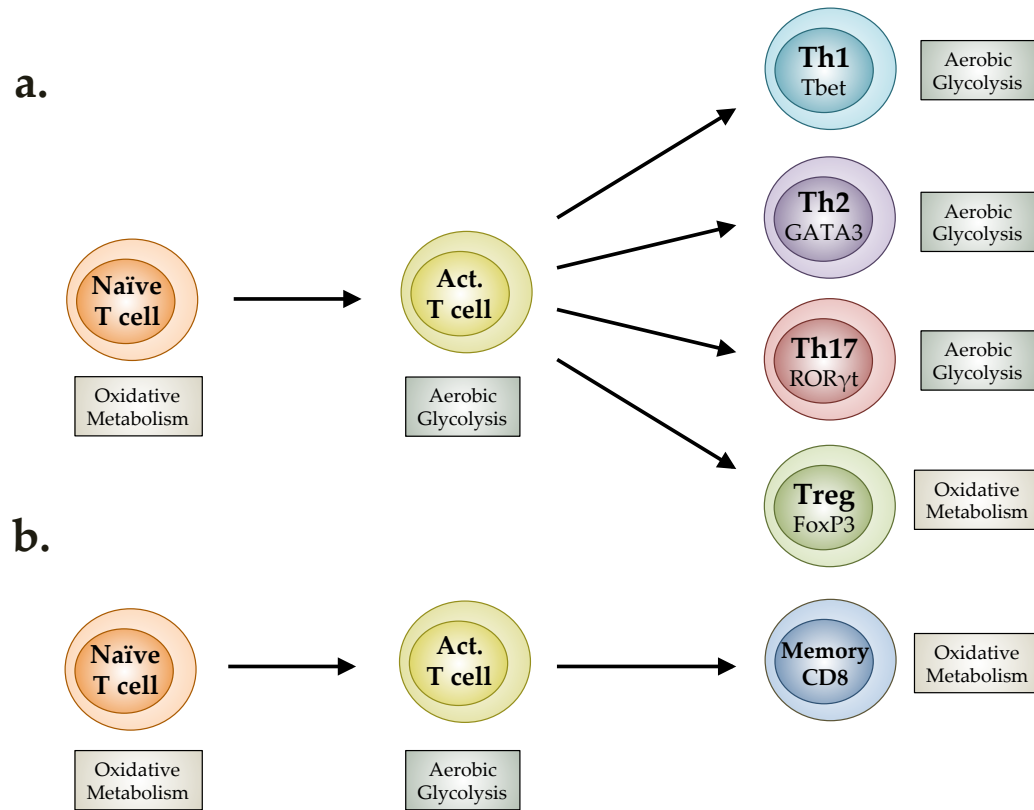


Figure 1.3: Distinct metabolic programs in the T cell subsets

As each of the T cell subsets has a unique role in the immune system, different types of T cells have unique metabolic programs and requirements. **(a)** Naïve CD4 T cells transition from an oxidative catabolic metabolism to an anabolic glycolytic metabolism. Depending on the cytokine environment during T cell activation, the T cell can then either differentiate into effector T cells (Th1, Th2 and Th17) with a glycolytic phenotype or regulatory T cells (Treg) that maintain and oxidative metabolism. **(b)** Similarly to CD4 T cells, naïve CD8 T cells start out in a quiescent oxidative state and transition to effector CD8 T cells, which utilize aerobic glycolysis. At the end of the immune response, CD8 T cells transition back to an oxidative metabolism to allow for the survival of long-lived memory T cells.

1.7 Metabolism in autoimmunity and inflammatory disease

The studies on T cell metabolism have largely come from *in vitro* experiments with culture media and supraphysiological T cell stimulation. Recent data have emerged, however, suggesting that this metabolic reprogramming is necessary to effector T cell function *in vivo*. For example, treatment of mice with 2-deoxyglucose to inhibit hexokinase and glycolysis alleviates EAE (Shi *et al.*, 2011). Inhibition of PDK1 with DCA treatment has also been shown to inhibit airway inflammation and collagen-induced arthritis in mice (Ostroukhova *et al.*, 2011; Bian *et al.*, 2009). In addition, T cells isolated from human asthma patients and treated with DCA had decreased proliferation and inflammatory cytokine production compared to vehicle (Ostroukhova *et al.*, 2011). Increased glucose metabolism has also been linked to autoimmune disease in Glut1 transgenic mice (Jacobs *et al.*, 2008). The increased expression of Glut1 in the T cells of these mice leads to increased rates of glucose uptake and glycolysis upon T cell activation. Aged Glut1 transgenic mice accumulated activated T cells in the spleen and lymph nodes and have anti-dsDNA antibodies and other signs of autoimmunity (Jacobs *et al.*, 2008; Michalek *et al.*, 2011b).

While much of the previous focus has been on glucose metabolism and aerobic glycolysis, mitochondrial dysfunction has also been associated with inflammatory disease, especially in the context of chronically activated T cells. For example, chronically stimulated T cells in the setting of graft-vs-host disease (GvHD) increase

both glycolysis and OXPHOS compared to normal T cells in response to alloantigen (Gatza *et al.*, 2011). Wahl *et al.* also found that autoreactive splenocytes in mice with systemic lupus erythematosus (SLE) preferentially oxidize glucose rather than convert it to lactate *in vivo* (Wahl *et al.*, 2010). Similarly, T cells from human lupus patients have increased mitochondrial mass and membrane potential compared to control T cells (Gergely *et al.*, 2002). These mitochondrial alterations may be used to specifically target autoreactive or chronically stimulated T cells. To this end, Gatza *et al.* showed that the treatment of T cells with the compound Bz-423, which inhibits F1F0-ATPase activity, decreased the viability of alloreactive but not healthy T cells (Gatza *et al.*, 2011). Treatment of mice with Bz-423 in the context of GvHD also improved the survival and clinical scores of the mice compared to vehicle control. This data suggests that targeting oxidative metabolism may be useful in certain contexts including alloreactive and chronically stimulated T cells.

In addition to cellular metabolism, the overall metabolic status of an organism may influence T cell function. Malnutrition has long been associated with immunosuppression, while obesity is classically associated with inflammation (Shears, 1991; Gregor *et al.*, 2011). Furthermore, the adipokine leptin is important to regulate energy intake and expenditure and has recently been shown to play a role in immune homeostasis. Leptin itself can function as a proinflammatory cytokine (Procaccini *et al.*, 2012). Further, both leptin-deficient humans and mice have immune defects including

reduced T cell numbers, decreased Th1 and increased Th2 cells (Mattioli *et al.*, 2005). Fasting-induced hypoleptinemia in mice also led to reduced cytokine production and decreased glucose uptake and glycolysis in T cells (Saucillo *et al.*, 2013). This was likely because leptin plays an important role to increase Glut1 and glucose metabolism. Leptin has also been shown to decrease Treg proliferation, likely through activation of the mTOR signaling pathway (Procaccini *et al.*, 2010). Therefore, leptin and other metabolic regulators can also contribute to T cell metabolism and differentiation.

1.8 Questions to be addressed

The metabolic reprogramming that occurs upon T cell activation has been well described (MacIver *et al.*, 2013; Gerriets *et al.*, 2012). Additionally, recent literature suggests that Treg utilize a distinct metabolic program from Teff (Michalek *et al.*, 2011a; Shi *et al.*, 2011). The metabolic manipulations used in these studies have pointed to metabolic differences between Teff and Treg, however the metabolic programs underlying each individual subset, Th1, Th2, Th17 and Treg, are unclear. Previous studies also use pharmacological agents to examine T cell metabolism, including 2-deoxyglucose and etomoxir, which inhibit glycolysis and fatty acid oxidation respectively.

The use of pharmacological agents complicates the interpretation of *in vivo* T cell data, as these inhibitors can affect all cell types, including antigen-presenting cells and

non-immune cells which may contribute indirectly to T cell function. In addition, pharmacological approaches can have off-target effects. Therefore, it is necessary to use a genetic approach in which metabolism can be altered specifically and intrinsically to the T cell to examine the affect of metabolic manipulation on T cell differentiation and function. Additionally, a more detailed understanding of these metabolic programs may provide a way to specifically modulate the immune system to favor one T cell subset over another. This would be useful in the context of autoimmune disorders. Currently, many autoimmune therapies affect all lymphocytes, leading to general immune suppression. Modulation of the balance between Th17 and Treg, specifically, would be advantageous in autoimmunity without causing unwanted side effects from general immune suppression.

Therefore, the work here examines a genetic approach of metabolic inhibition through Glut1 deletion. As described above, Glut1 is one of the major glucose transporters in T cells and is important to control the rate of glucose uptake and glycolysis upon T cell activation (Jacobs *et al.*, 2008; Michalek *et al.*, 2011a). Previous work has shown that effector T cells (Th1, Th2 and Th17) express much higher levels of Glut1 than Treg, however the role of Glut1, and importantly, the cell-intrinsic role of glucose metabolism in T cell differentiation and inflammation was not directly tested (Michalek *et al.*, 2011a).

Additionally, previous literature has shown broad metabolic differences between Teff and Treg, however the metabolic phenotypes of both Teff and Treg are poorly understood. Here, Teff and Treg metabolism is explored to determine the metabolic programs underlying each of the T cell subsets and test if dependence on distinct metabolic pathways will allow selective targeting of different T cell populations. A question that arises from differences in T cell metabolic profiles is how and why this occurs. What accounts for differences in fuel utilization and metabolic pathways? Particularly, Treg seem to not rely on glucose but instead have fuel flexibility and remain more oxidative than Teff. Because the transcription factor FoxP3 drives the Treg program, the role of FoxP3 in metabolism is also examined to determine if FoxP3 drives the Treg metabolic program. Overall, the work presented here examines the metabolic programs and regulation of the CD4 T cell subsets and potential metabolic targets that could be used to treat autoimmune and inflammatory conditions.

2. Materials and Methods

2.1 Mice

Six to eight week old C57BL/6J mice from Jackson Laboratory were used for all experiments unless otherwise indicated. *Glut1^{fl}* animals were crossed to mice expressing Cre recombinase under the control of the *p56Lck*, *CD4* or *UbiCreER* promoters (Jackson Laboratory) and have been described previously (Young *et al.*, 2011). Mice expressing endogenous levels of myc-epitope tagged Glut1 (*Glut1^{myc}*) have also been described (Michalek *et al.*, 2011a). All procedures were performed under Duke University Medical Center IACUC-approved protocols.

2.2 T cell isolation and differentiation

Naïve CD4⁺CD25⁻ T cells were isolated *ex vivo* and T helper cell subsets were generated as described previously (Michalek *et al.*, 2011a). Briefly, CD4⁺CD25⁻ T cells were cultured on irradiated splenic feeder cells (300 Gy) with 2.5µg/mL of anti-CD3 antibody at a ratio of 5:1 in RPMI supplemented with 10% FBS, sodium pyruvate, penicillin/streptomycin, HEPES and beta-mercaptoethanol. The following cytokines were added to each subset: Th1, 10ng/mL IL-12 (R&D Systems), 10µg/mL anti-IL-4 (eBioscience, clone 11B11), 1µg/mL anti-IFNγ (eBioscience); Th2, 1000U/mL recombinant IL-4 (R&D Systems), 10µg/mL anti-IL-12 (eBioscience), 10µg/mL anti-IFNγ; Th17,

20ng/mL IL-6 (R&D Systems), 2.5ng/mL TGF β (R&D Systems), 10 μ g/mL anti-IFN γ ; Treg, 3ng/mL TGF β . On day 3 post stimulation, cells were split 1:2 and re-plated with IL-2 alone for an additional 2 days. In some experiments, cells were treated with 5nM rotenone, 500 μ M 2-deoxyglucose, 10mM dichloroacetate or labeled with CellTrace Violet (Invitrogen) per manufacturer's instructions.

2.3 Metabolic assays

Glycolysis and glucose uptake assays using ^3H -glucose or ^3H -2-deoxyglucose have been described previously (Plas *et al.*, 2001; Wieman *et al.*, 2007). Glutamine oxidation, pentose phosphate pathway (PPP) flux and glucose oxidation were determined by the rate of $^{14}\text{CO}_2$ release from U- ^{14}C -glutamine, 1- ^{14}C -glucose and 6- ^{14}C -glucose as described (Wang *et al.*, 2011). All values were normalized to cell number. OCR and ECAR were measured with an XF24 extracellular flux analyzer (Seahorse Bioscience) as described (Wu *et al.*, 2007). Suspension cells were attached to culture plates using Cell-Tak (BDBioscience). OCR and ECAR were measured in unbuffered RPMI (Sigma-Aldrich) supplemented with 10mM D-glucose (Sigma-Aldrich), 10mM L-glutamine, and 10mM sodium pyruvate, as indicated. OCR and ECAR values were normalized to cell number. For certain experiments, ECAR was measured over time following injection of 10mM D-glucose, oligomycin and 2DG. Glycolytic capacity is defined as the difference between the ECAR following the injection of oligomycin and

basal ECAR. Spare respiratory capacity is defined as the percent increase in OCR between the initial basal readings and the injection of FCCP. Lactate production was measured by colorimetric assay.

2.4 Flow cytometry

Murine T cells were labeled with anti-mouse CD4-allophycocyanin (APC), CD4-fluorescein isothiocyanate (FITC), CD4-eFluor 450, CD25-phycoerythrin (PE), CD8 PE-Cy5.5, CD44-Violet Blue, CD71-APC, CD98-PE or Thy1.2-FITC (all eBioscience). Exofacially tagged Glut1^{myc} was stained with mouse anti-myc (Millipore) followed by rat anti-mouse IgG-PE (eBioscience). To measure intracellular cytokines, cells were unstimulated or stimulated for 5h with PMA (50ng/ml; Sigma-Aldrich) and ionomycin (750ng/ml; Calbiochem) in the presence of GolgiStop (IL-4, IL-17) or GolgiPlug (IFN γ , IL-17, IL-2), permeabilized with Cytofix/Cytoperm Plus (BDBiosciences), and then stained with IFN γ -APC, IL-2-PE, IL-17-PE, or IL-4-APC (all eBioscience). FoxP3 staining was performed using FoxP3-PE in combination with Regulatory T cell staining kit #1 (eBioscience). Intracellular mouse and human Glut1 staining was performed by fixing cells in 1% paraformaldehyde, permeabilizing with methanol and then staining with rabbit anti-Glut1 (Abcam) followed by rat anti-rabbit IgG-PE or rat anti-rabbit IgG-APC (eBioscience). Cell proliferation was assayed by flow cytometry of carboxyfluorescein succinimidyl ester (5 μ M CFSE; Molecular Probes) labeled cells. Cell viability was

determined flow cytometrically by propidium iodide exclusion (1µg/ml PI; Invitrogen). Data were acquired on a MacsQuant cytometer (Miltenyi Biotec) and analyzed with FlowJo software (TreeStar).

2.5 Treg suppression assay

Treg cells were differentiated as described above and cultured at ratios of 1:1, 1:2, 1:4 or 1:8 with CFSE labeled CD4⁺CD25⁻ T cells on plates coated with 5µg/ml of anti-CD3 and anti-CD28 (eBioscience). Treg suppression of CD4 T cell proliferation was determined 72h post stimulation by CFSE dilution of target population.

2.6 Glucose transporter family expression

RNA was isolated from snap-frozen cell pellets using RNAeasy Plus Mini kit (Qiagen). Glucose transporter absolute copy number was determined as described (Rudolph *et al.*, 2011). Briefly, total RNA was converted to cDNA using the Verso cDNA synthesis kit with blended oligo dT and random hexamer primers (ThermoFisher). qPCR was performed using Absolute Fast qPCR Mix-lox Rox (ThermoFisher). Probe sets (Applied Biosystems) and amplicon sizes are given in Supplementary Table 1. Data were collected with the 7500 fast thermocycler (Applied Biosystems), and the Ct value for unknowns were fitted to each target regression curve to quantify transcript copy number for that target.

2.7 Semi quantitative real time PCR

RNA was extracted from cells with the RNeasy RNA purification minikit (Qiagen). Reverse-transcription PCR was performed with iScript cDNA synthesis kit (Biorad). Real-time PCR was performed with iQSYBR Green detection chemistry (Biorad) using an iCycler (BioRad). Relative mRNA levels were normalized to 18S RNA content. Primers used: 18S (Fwd:5'GTAACCCGTTGAACCCCAT3' Rev:5'CCATCCAATCGGTAGTAGCG3'), murine Glut1 (Fwd:5'AGCCCTGCTACAGTGTAT3' Rev:5'AGGTCTCGGGTCACATC3').

2.8 Immunoblotting

Immunoblotting was performed as described previously (Jacobs *et al.*, 2008). Blots were probed for CPT1 α (Proteintech group, 15184-1-AP), Glut1 (abcam, ab115730) or β -actin (Sigma, A5441), followed by mouse- or rabbit-conjugated horseradish peroxidase (HRP) (Cell Signaling Technology). HRP-conjugated antibodies were detected by enhanced chemiluminescence detection (Thermofisher). Alternatively, primary antibodies were followed by fluorescently labeled anti-mouse or rabbit antibodies (LiCor) and imaged using the Odyssey infrared imaging system (LiCor). This included the following antibodies: Glut1 (ab652, Abcam), Glut3 (Millipore), Glut6 (Osenses), Glut8 (Millipore), Glut9 (Thermo-Fisher Scientific), Hk1 (Millipore), Hk2

(Millipore), Hk3 (Abcam), Hk4 (Abcam), OXPHOS antibody cocktail (Abcam), CPT1 α , cytochrome C (BD Biosciences), Actin (Abcam).

2.9 FoxP3-ER cell lines

FL5.12 cells were infected with control or FoxP3-ER expressing lentivirus and selected with NGFR. The FoxP3-ER-NGFR construct has been previously published (Allan *et al.*, 2008). Three clones of each were selected based on equivalent NGFR expression. To activate FoxP3-ER, 100nM 4-hydroxtamoxifen (4-OHT) was added 36 hours prior to experimental procedures.

2.10 Retroviral FoxP3 expression

CD4⁺CD25⁻ primary T cells were isolated and activated with 3ng/mL PMA and 1 μ M ionomycin overnight. Cells were then infected with FoxP3-NGFR or NGFR control retrovirus along with 8 μ g/mL polybrene and centrifuged for 90 min at 2500rpm. Cells were rested for 5h and then plated with fresh media and 10U/mL IL-2 for 72h. FoxP3-NGFR or NGFR control cells were positively selected with PE magnetic beads following NGFR-PE labeling (\geq 90% purity, Miltenyi Biotec).

2.11 T cell transfer model of colitis

Splenic CD4 T cells were isolated as described above, and naïve effector (CD4⁺CD25⁻CD45RB^{hi}) and regulatory (CD4⁺CD25⁺CD45RB^{lo}) T cells were sorted (FACSvantage, BDBioscience). Naïve effector T cells from were injected i.p. into 6-8 week old C57BL/6 RAG1^{-/-} recipients (4x10⁵ cells/mouse). Treg cells (2x10⁵ cells/mouse) were co-injected as indicated. Alternatively, Treg were injected 21 days later after disease initiation where indicated. Because the mice were *H. pylori* negative, and colitis does not occur spontaneously in this setting, disease was initiated two weeks following T cell injection with 200ppm piroxicam (Sigma-Aldrich) in powdered rodent chow for 5 days to enhance mucosal exposure to enteric bacteria and induce colitis (Hale *et al.*, 2005). In certain cases, mice were then injected i.p with 4mg/kg/day of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) for 4 days to induce *Glut1*ⁿ deletion and monitored three times weekly. For analysis the colon was divided into 5 segments representing the cecum, and proximal, mid-, distal, and terminal colon/rectum. Tissues were fixed and a board-certified pathologist blindly scored the severity of colonic inflammation in hematoxylin and eosin-stained sections as described (Hale *et al.*, 2005).

2.12 Ova Immunization Model

Ovalbumin (Ova)-specific OT-II T cell receptor (TCR) Thy1.2 transgenic control ($CD4^{Cre}Glut^{+/+}$) or knockout ($CD4^{Cre}Glut1^{fl/fl}$) CD4 T cells were labeled with CTV and adoptively transferred into wild type Thy1.1 hosts. One day later, mice were immunized with i.p. Ova in Complete Adjuvant (Sigma). Three days after immunization, lymphocytes were isolated and proliferation was assessed by CTV dilution of Thy1.2 labeled cells.

2.13 PCR arrays

RNA was isolated from naïve, Th1, Th2, Th17 or Treg cells using RNeasy Plus Mini kit (Qiagen) following the manufacturers' instructions. 1 µg total RNA was subjected to single-strand cDNA synthesis using the RT2 first strand kit (Qiagen). The cDNA was used in mitochondrial energy metabolism and glucose metabolism SuperArray RT2 Profiler PCR arrays were used according to the manufacturer's instructions and assayed on a ViiA 7 (Applied Biosystems). Data was analyzed using the RT2 Profiler program supplied by Qiagen and normalized to the housekeeping genes TATA box binding protein and Beta-glucuronidase as determined by GeNorm in accordance with the MIQE Guidelines.

2.14 Human T cell isolation and siRNA

Peripheral blood mononuclear cells (PBMC) were isolated from healthy, anonymized donors by density gradient centrifugation. T cells were isolated by magnetic bead negative selection ($\geq 90\%$ purity; Miltenyi Biotec) and cultured in RPMI 1640 (MediaTech) supplemented with 10% FBS (Gemini Bio-Products), L-glutamine (Gibco), penicillin-streptomycin (Gibco), and 50 μ M β -mercaptoethanol (β -ME) (Sigma-Aldrich). Human siRNA pools (Dharmacon: FoxP3 or scrambled pool) were transiently transfected by nucleofection (Amaxa; Human T cell Nucleofector kit program U14; Lonza) and cells were rested 4-6h before stimulation. Where indicated cells were activated on plates coated with 5 μ g/ml anti-CD3 (clone UCHT1) and 5 μ g/ml anti-CD28 (eBioscience) and stimulated in the presence of 20ng/ml IL-2 (Novartis).

2.15 Lentiviral PDHK1 shRNA

PDHK1 shRNA expressing and control lentiviruses were purchased from Sigma. CD4 T cells were isolated and differentiated as described above and infected with PDHK1 shRNA or control lentivirus after 24 hours. Polybrene was added to facilitate the infection and the cells were centrifuged for 90 min at 2500rpm. After 2 days, the cells were split 1:2 and treated with 2 μ g/mL puromycin for 2 days to select for infected cells.

2.16 Experimental autoimmune encephalomyelitis (EAE)

EAE was induced as previously described (Michalek *et al.*, 2011b). Briefly, wildtype mice were injected with 100 ng MOG peptide mixed with Complete Freund's Adjuvant and Mycobacterium tuberculosis followed by 1 µg/mL pertussis toxin administered by i.p injection on days 0 and 2. DCA (2g/L) was given to the mice in their drinking water where indicated. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tone in the tail; 2, hindlimb paresis; 3, hindlimb paralysis; 4, tetraplegia.

2.17 Statistical analysis

Statistical analyses were performed with Prism software (GraphPad) using the nonparametric Mann Whitney test. The Wilcoxon signed-rank test was used for paired samples. Longitudinal data was analyzed by two-way ANOVA followed by Tukey's test. Statistically significant results are indicated (* $p < 0.05$) and n.s. indicates select non-significant data.

3. The Glucose Transporter Glut1 is Essential for Activation and Effector, but Not Regulatory, CD4 T Cell Metabolism and Inflammation

3.1 Introduction

T cell activation initiates a transition from quiescence to rapid cell growth, proliferation, and differentiation into functional subsets to drive or suppress the immune response (Zhu *et al.*, 2010). Effector CD4 T cells (Teff; including Th1, Th2, and Th17) promote immunity and are enriched in inflammatory diseases. Regulatory CD4 T cells (Treg), in contrast, suppress immunity and are decreased in number or function in these settings (Zhu *et al.*, 2010). Importantly, the transition from quiescence to rapid growth and proliferation increases energetic and biosynthetic demands. Activated T cells thus upregulate nutrient uptake and metabolic rates (MacIver *et al.*, 2013), resulting in a significant elevation of glucose and amino acid transport (Frauwirth *et al.*, 2002; Sinclair *et al.*, 2013; Wang *et al.*, 2011) that may provide new directions to modulate immunity. T cell metabolism has been shown in distinct settings to require lipid synthesis (Kidani *et al.*, 2013) or oxidation (Byersdorfer *et al.*, 2013; Gatzert *et al.*, 2011), mitochondrial reactive oxygen species (Sena *et al.*, 2013), and amino acid uptake (Sinclair *et al.*, 2013). However, the *in vivo* mechanism and roles of increased glucose uptake and metabolism in T cell-mediated inflammatory diseases remain uncertain.

It is now evident that metabolic reprogramming is shaped to support specific cell functions (MacIver *et al.*, 2013). *In vitro* generated Teff strongly induce glycolysis and decrease lipid oxidation (Michalek *et al.*, 2011a; Shi *et al.*, 2011; Wang *et al.*, 2011). In contrast, *in vitro* induced Treg and memory CD8 T cells utilize lipid oxidation as a primary metabolic pathway (Michalek *et al.*, 2011a; Pearce *et al.*, 2009; Shi *et al.*, 2011). These metabolic programs provide distinct metabolites (MacIver *et al.*, 2013), signaling through the mTORC1 pathway (Sinclair *et al.*, 2013), and cytokine production (Cham and Gajewski, 2005; Chang *et al.*, 2013). Importantly, induced Teff and Treg may be differentially sensitive to glycolytic inhibition, as *in vitro* glucose limitation or 2DG treatment suppressed Th17 but not Treg cells (Michalek *et al.*, 2011a; Shi *et al.*, 2011). Because these pharmacologic approaches result in broad non-specific effects that impact all cells, mechanistic insight has been limited. A genetic approach is needed to establish the cell-intrinsic roles of glucose metabolism in T cell activation and regulation of inflammation.

Glucose uptake provides a key metabolic control point through the Glut family of facilitative glucose transporters. The fourteen Glut family members are differentially regulated and possess distinct substrates and biological properties (Thorens and Mueckler, 2010). The array of Glut transporters utilized by T cells in activation and differentiation has not yet been defined. *In vitro* stimulated murine and human T cells express high levels of Glut1 (Slc2a1) (Frauwirth *et al.*, 2002; Jacobs *et al.*, 2008) and Teff

cells maintain higher levels of Glut1 than Treg (Michalek *et al.*, 2011a). Glut1 can promote Teff, as transgenic Glut1 overexpression selectively increased Teff frequency and led to inflammatory disease (Jacobs *et al.*, 2008; Michalek *et al.*, 2011a).

Here we examine the Glut transporter family to directly test the role and mechanisms that control T cell glucose uptake and metabolism in activation and in inflammatory disease. Although T cells expressed four distinct glucose transporter isoforms, genetic targeting showed a selective role for Glut1 in proliferative thymocytes and Teff cells. While resting murine and human T cells were independent of Glut1, Teff required Glut1 for efficient expansion and specification *in vivo*. In contrast, both natural and induced Treg were independent of Glut1. As a result, Glut1-deficient Teff were unable to effectively induce either Graft-vs-Host Disease (GvHD) or colitis, while Treg could protect against colitis independent of Glut1. Thus, Glut1 has a selective cell-intrinsic function in T cell metabolic reprogramming to drive glycolysis of Teff for growth, expansion, and inflammatory disease.

3.2 Results

3.2.1 T cells express a subset of dynamically regulated Glut family transporters

The mechanism of glucose uptake and role of Glut family glucose transporters in activation-induced glucose uptake in T cells has not been directly established. The absolute expression of each Glut family member was, therefore, mRNA transcript copy

number was quantified in resting and activated murine T cells (**Fig. 3.1A**). Of the thirteen glucose transporter family members measured, only Glut 1, 3, 6, and 8 were detected. Slc2a1 (Glut1) and Slc2a3 (Glut3) mRNA were equally expressed in resting CD4 T cells. Following activation, Slc2a1 (Glut1) was induced or sustained, while Slc2a3 (Glut3) mRNA became less prominent. Slc2a6 (Glut6) was also induced with activation, but remained at lower copy number than Slc2a1 (Glut1). Glut family member expression was also measured in induced Th1, Th2, Th17, and Treg (**Fig. 3.1B**). Again, Gluts 1, 3, 6, and 8 were the only detectable Glut transporters, and while each T cell subset had a distinct transporter profile, Glut1 was found in the highest copy number in each case. Of note, differentiated cells expressed Glut3 more similarly to Glut1.

In addition to mRNA levels, the trafficking of Glut1 to the cell surface is also highly regulated (Edinger and Thompson, 2002; Wieman *et al.*, 2007). Glut1 protein levels and trafficking were therefore examined during T cell activation. Because available antibodies react poorly with the extracellular domains of Glut1, Glut1^{myc} knock-in mice were used to measure cell surface Glut1 in T cell development and activation. In this model a tandem-Myc tag was knocked into exon 3 of Slc2a1, which encodes the large exofacial loop of Glut1, enabling sensitive and specific flow cytometric measurement of endogenous cell surface Glut1 using antibodies against the Myc epitope (Michalek *et al.*, 2011a).

Increased total intracellular expression of Glut1^{myc} was detected within two hours of CD3 and CD28 stimulation. Cell surface levels of Glut1^{myc} increased more slowly, but elevated Glut1^{myc} surface expression were measurable within four hours of stimulation and maximal after 24 hours (**Fig. 3.1C**). Consistent with previous data implicating the PI3K-Akt-mTORC1 pathway in the regulation of Glut1 expression and trafficking (Frauwirth *et al.*, 2002; Wieman *et al.*, 2007), inhibitors of PI3K (LY294002), PI3K/mTOR kinase (PP242), or mTORC1 (rapamycin) suppressed the induction of cell surface Glut1 (**Fig. 3.1D**). Glut1 upregulation and cell surface trafficking is, therefore, an early event in T cell activation mediated in part through PI3K-Akt-mTORC1 signaling.

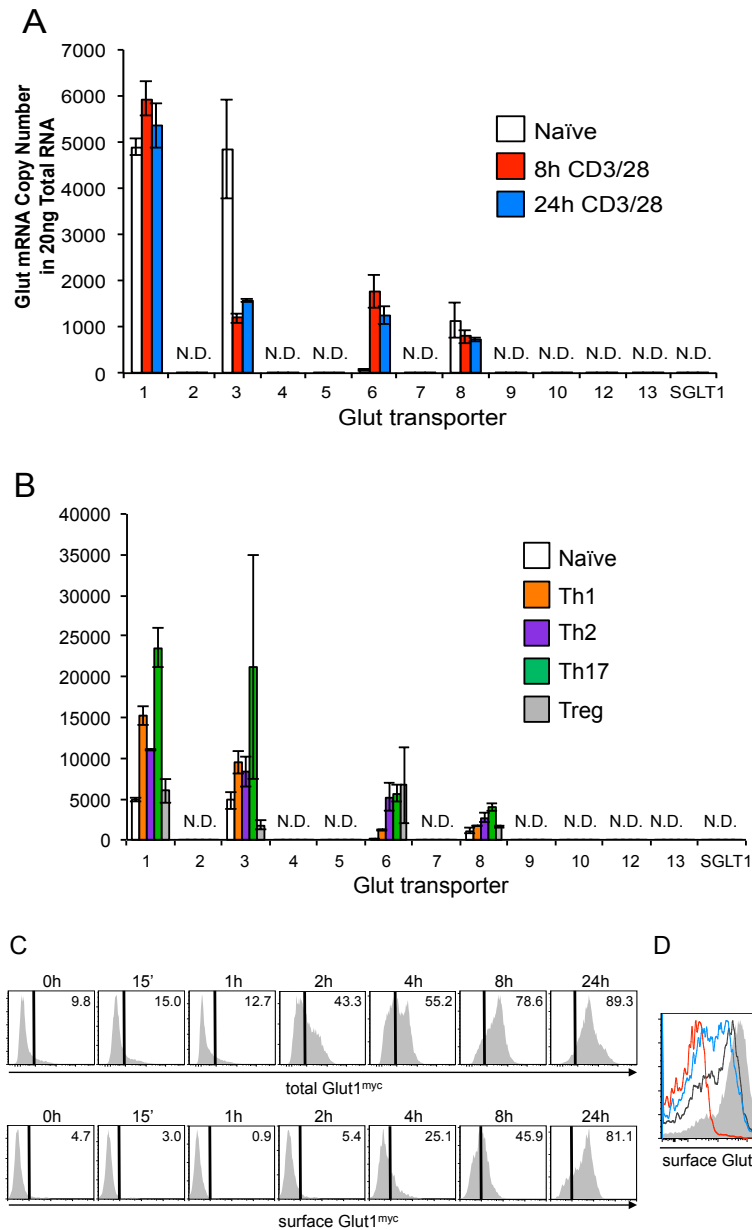


Figure 3.1: Glut1 is selectively and rapidly increased in murine T cell activation.

(A, B) Glut family mRNA copy number in (A) naïve and CD3/CD28-stimulated CD4 murine T cells, and (B) *in vitro* polarized CD4 T cell subsets. N.D.: not detected.

(C, D) Glut1myc expression in CD3/CD28 stimulated CD4 Glut1^{myc} T cells (C) over time and (D) with inhibitors or vehicle control. Mean \pm SD from 3 or more independent experiments are shown. MC Rudolph and SM Anderson: A, B; AN Macintyre: C, D.

3.2.2 Glut1 is required for growth and proliferation of mature T cells

Based on the dynamic expression of Glut1, the specific role of this transporter was tested in T cell development and activation. Glut1^{fl/fl} mice (Young *et al.*, 2011) were crossed with LckCre transgenic mice to specifically delete Glut1 in early DN thymocytes. Total thymocyte numbers were reduced 60-70% in LckCreGlut1^{fl/fl} mice compared to control animals (data not shown). To bypass potential developmental defects, Glut1^{fl/fl} mice were crossed to CD4Cre transgenic mice, which delete loxP flanked genes in the DP to SP transition following the proliferative DN3-DN4 stage in thymopoiesis. Thymocyte numbers and phenotype were normal in CD4CreGlut1^{fl/fl} mice (data not shown). Additionally, resting peripheral T cells do not require Glut1 for survival.

The decreased numbers of LckCreGlut1^{fl/fl} thymocytes after the proliferative DN3-DN4 phase and apparent Glut1-independence of resting T cell survival suggested that Glut1 is selectively required to support proliferation. To test the dependence of peripheral T cells on Glut1 for activation-induced proliferation, Ovalbumin (Ova)-specific OT-II T cell receptor (TCR) transgenic control or CD4CreGlut1^{fl/fl} CD4 T cells were labeled with the proliferation dye CellTrace Violet (CTV) and adoptively transferred into Thy1.1 hosts that were then immunized with Ova. Importantly, Glut1-deficient CD4 T cells had reduced proliferation (**Fig. 3.2A**). We also tested the ability of Glut1-deficient T cells to undergo homeostatic proliferation after adoptive transfer into irradiated and lymphopenic recipients. Consistent with a need for Glut1 to allow

proliferation *in vivo*, Glut1-deficient CD8 and CD4 T cells underwent only limited homeostatic proliferation (**Fig. 3.2B**).

T cells were stimulated *in vitro* to examine mechanisms that suppressed proliferation and accumulation of Glut1-deficient T cells *in vivo*. Importantly, *in vitro* stimulated Glut1-deficient T cells grew very poorly, as evidenced by a failure to increase forward light scatter by flow cytometry (**Fig. 3.2C**). Further, activation sufficient to drive the proliferation of control T cells failed to induce proliferation (**Fig. 3.2D**) and instead led to rapid induction of cell death of many LckCreGlut1^{fl/fl} T cells (**Fig. 3.2E**). Glut1-deficient T cells that survived this initial period of activation appeared under metabolic stress and had increased levels of phospho-AMPK (**Fig. 3.2F**) and failed to sustain activated mTORC1 signaling, as assessed by phosphorylation of the downstream p70 S6 kinase target, small ribosomal subunit S6 (**Fig. 3.2G**). The mTOR pathway can broadly regulate nutrient uptake (McCracken and Edinger, 2013), and the transferrin receptor (CD71) and the 4F2hc amino acid transporter (CD98) were not efficiently induced in activated Glut1-deficient T cells (data not shown). Most signaling pathways, however, were unchanged, as cMyc was induced and phospho-Akt and -ERK were equivalent or only modestly reduced and activation markers were normal in activated Glut1-deficient T cells (data not shown).

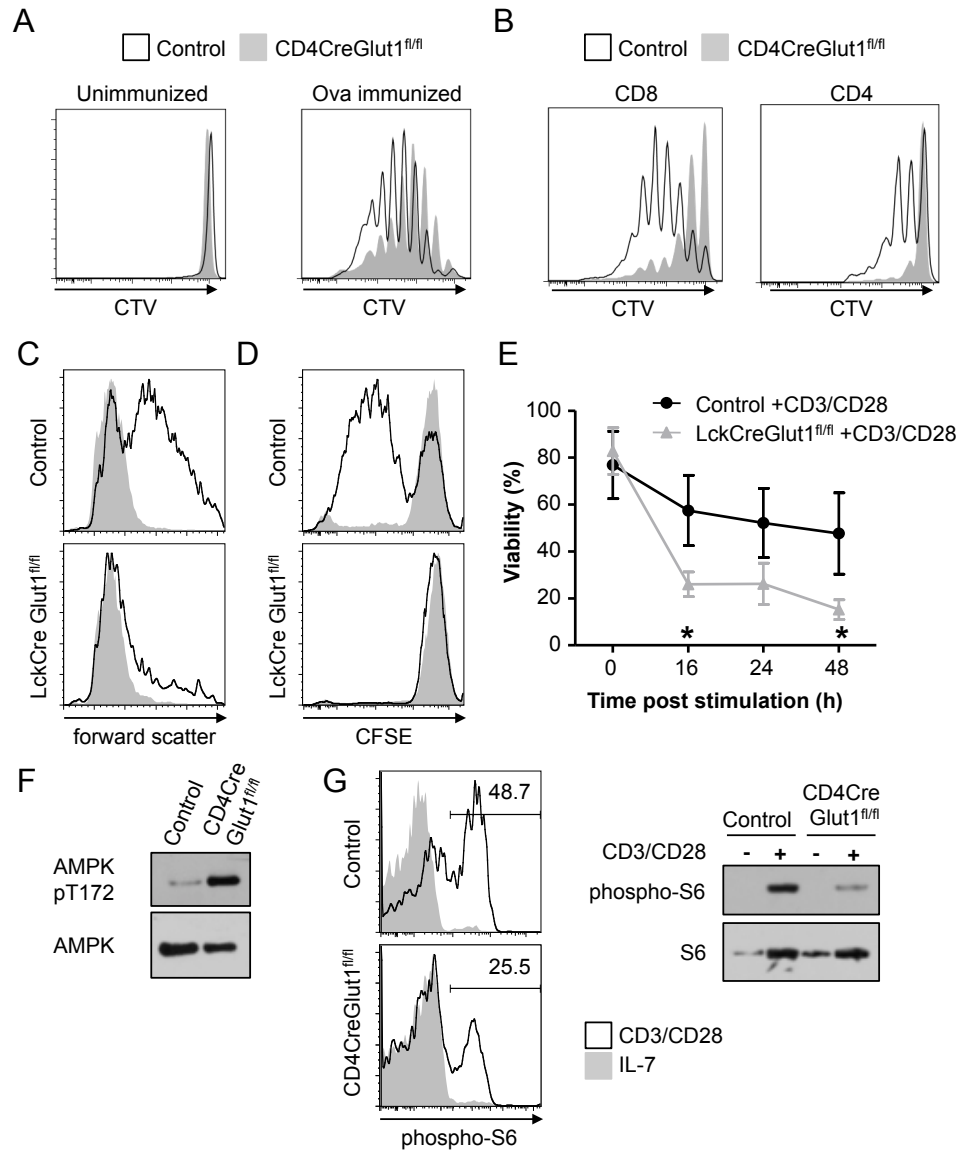


Figure 3.2: Glut1 is necessary to support activation-induced growth, proliferation and survival

(A, B) Proliferation of CellTrace Violet (CTV) labeled control (Glut1^{fl/fl}) and CD4CreGlut1^{fl/fl} (A) OT-II transgenic T cells on day 3 after adoptive transfer \pm immunization with Ovalbumin or (B) T cells 6 days after adoptive transfer into intact or irradiated recipients for homeostatic proliferation. (C) Control (Glut1^{fl/fl}) and LckCreGlut1^{fl/fl} T cells were rested in IL-7 or CD3/CD28- and cell size (forward scatter) of viable cells was determined by flow cytometry after 24h. (D, E) Control (Glut1^{fl/fl}) and LckCreGlut1^{fl/fl} T cells were CFSE-labeled and either rested in IL-7 or CD3/CD28-

stimulated and examined by flow cytometry for (D) proliferation at 72 hours or (E) viability over time. (F) Control (Glut1^{fl/fl}) and CD4CreGlut1^{fl/fl} T cells were CD3/CD28-stimulated 16h and analyzed by immunoblot. (G) Control (Glut1^{fl/fl}) and CD4CreGlut1^{fl/fl} T cells were rested in IL-7 or CD3/CD28-stimulated for 10h and analyzed by intracellular flow cytometry and immunoblot. Data are representative of n=3 mice/group (A, B) a minimum of (F, G) 2 or (C-E) 3 experiments. (E) Shows mean \pm SD of 3 independent experiments. AN Macintyre: C-G.

3.2.3 Glut1 is necessary to support T cell metabolic reprogramming upon activation

Glucose uptake and glycolysis were measured in resting and activated T cells to assess the metabolic role of Glut1. Consistent with a selective role in activation, resting IL-7 treated peripheral T cells did not rely on Glut1 and had similar rates of glucose uptake, glycolysis, and lactate production regardless of Glut1 expression (**Fig. 3.3A, B, C**). However, while control T cells rapidly increased glucose metabolism after activation, stimulated LckCreGlut1^{fl/fl} T cells maintained only a basal rate of glucose uptake and greatly reduced glycolytic rate (**Fig. 3.3A, B**). Likewise, stimulated CD4CreGlut1^{fl/fl} T cells failed to increase lactate production when measured directly or by extracellular acidification rate (ECAR) (**Fig. 3.3C, D**). As a consequence, activated Glut1-deficient T cells had reduced glycolytic capacity (**Fig. 3.3E**) and an elevated ratio of oxygen consumption rate (OCR) to ECAR (**Fig. 3.3F**). Glut1 is, therefore, essential for rapid metabolic reprogramming to aerobic glycolysis for maximal growth, survival, and proliferation of in vitro stimulated T cells.

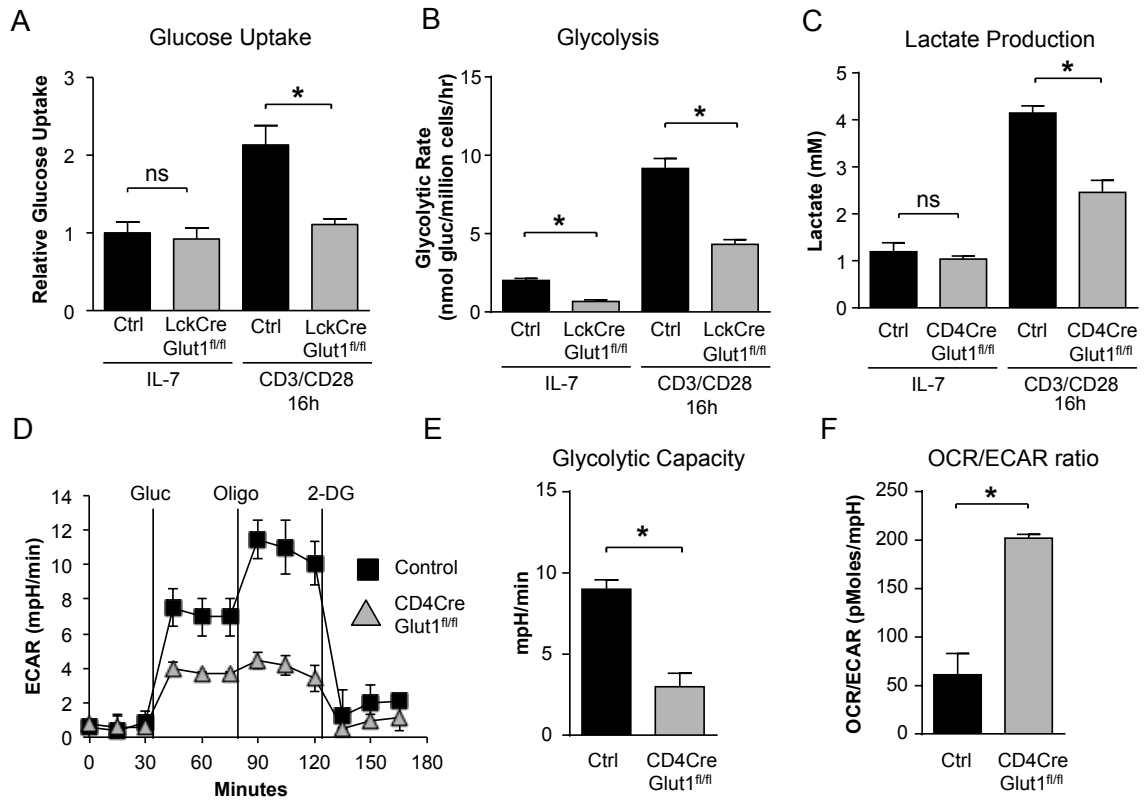


Figure 3.3: Glut1 is required for activation-induced metabolic reprogramming

(A, B) Control (Glut1^{fl/fl}) and LckCreGlut1^{fl/fl} T cells were rested in IL-7 or CD3/CD28-stimulated and (A) glucose uptake or (B) glycolytic rate was measured after 16h. (C-F) Control (Glut1^{fl/fl}) and CD4CreGlut1^{fl/fl} T cells were rested in IL-7 or CD3/CD28-stimulated for 16h. (C) Total lactate produced was measured. (D) Extracellular acidification rate (ECAR) was assessed after the addition of glucose (gluc), oligomycin (oligo), and 2-deoxyglucose (2-DG) at indicated times and (E) glycolytic capacity and (F) Oxygen Consumption Rate (OCR)/ECAR ratio determined. Mean \pm SD (n=4) are shown from a minimum of 2 or more independent experiments. AN Macintyre: A.

3.2.4 Glut1 is required for Teff, but not nTreg or iTreg, generation

We and others have previously shown *in vitro* that murine Teff (Th1, Th2, and Th17) utilize a highly glycolytic metabolism while Treg are primarily oxidative and use lipids as a fuel (Michalek *et al.*, 2011a). Control and Glut1-deficient Teff and Treg were therefore examined to test the selective dependence of CD4 subsets on Glut1.

Interestingly, while overall peripheral T cell numbers and frequency were lower in the spleen of LckCreGlut1^{fl/fl} mice, the CD4⁺ FoxP3⁺ natural Treg (nTreg) population was not decreased and only FoxP3 negative cells were reduced (**Fig. 3.4A**). This selective loss of FoxP3⁻ cells led to an increase in FoxP3⁺ nTreg in the peripheral CD4 T cell compartment (**Fig. 3.4B**). Naïve CD4 T cells can be induced to differentiate into Th1, Th2, Th17, or Treg subsets in appropriate cytokine conditions (Zhu *et al.*, 2010). CD4 T cells from control and CD4CreGlut1^{fl/fl} mice were therefore activated *in vitro* under polarizing conditions to generate Teff and Treg. The addition of cytokines reduced activation-induced cell death, allowing generation of each subset. The surviving cells in each case remained Glut1-deficient (**Fig. 3.4C**). Importantly, the viable cell number in each Teff culture and the fraction of viable cytokine producing cells in Th1 and Th17 cultures were reduced by Glut1-deficiency (**Fig. 3.4D**). Conversely, induced Treg cultures were unaffected by Glut1-deletion and maintained normal cell numbers, fraction positive for FoxP3, and ability to suppress Teff proliferation (**Fig. 3.4D, E**).

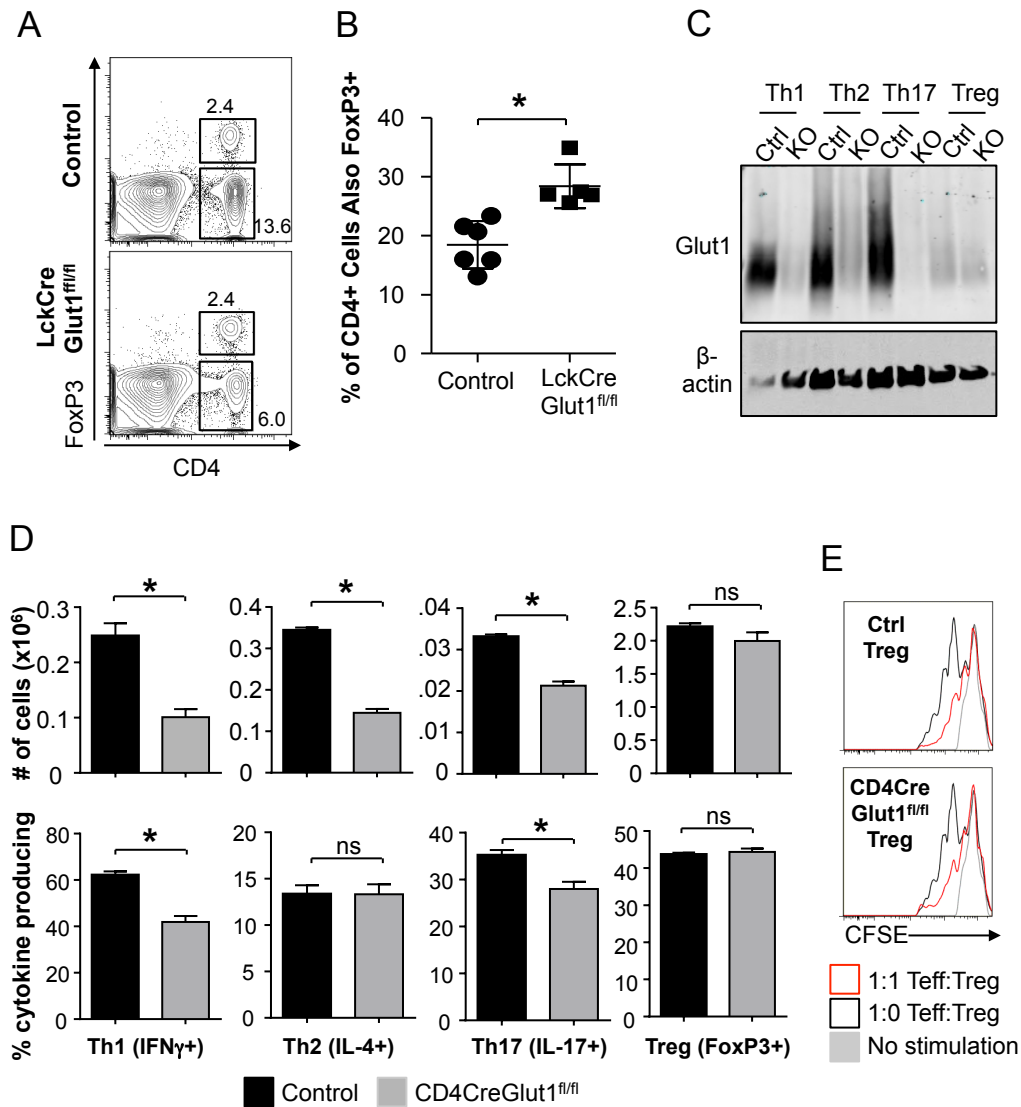


Figure 3.4: Glut1 is required for Teff, but not Treg, generation or function

(D, E) Flow cytometry of control (Glut1^{fl/fl}) and LckCreGlut1^{fl/fl} spleen for FoxP3⁺ CD4 T cells. (D) Representative plot and (E) cumulative data of fraction of CD4 T cells expressing FoxP3. (F-H) Th1, Th2, Th17, and Treg were induced using control (Glut1^{fl/+}, Glut1^{fl/fl}) and CD4CreGlut1^{fl/fl} CD4 T cells and analyzed by (F) immunoblot, (G) flow cytometry to determine the number of live skewed cells and percentage of live cells expressing subset markers. (H) Treg function was tested in an *in vitro* suppression assay. Data are representative or show mean cell count \pm SD from (D, E) 5, (F-H) 3, or (A-C) 2 independent experiments.

The normal number and function of Glut1-deficient Treg did not appear due to compensation by alternate glucose transporters, as Gluts 3 and 8 were unchanged and expressed at low levels and Glut6 was only modestly increased by Glut1-deletion (Fig. 3.5). Rather, these data suggest that Teff, but not Treg, are Glut1-dependent.

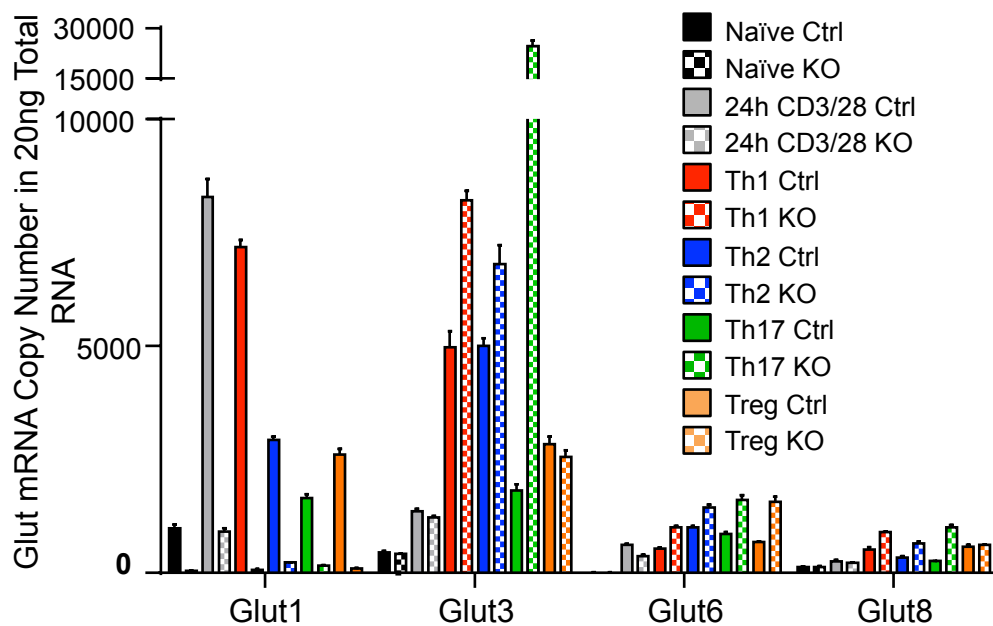


Figure 3.5: Glut transporter compensation for Glut1 loss in T cell subsets

CD4 T cells from control ($Glut1^{fl/+}$, $Glut1^{fl/fl}$) and $CD4CreGlut1^{fl/fl}$ mice were collected *ex vivo* (naïve), stimulated for 24 hours (24h CD3/CD28) or polarized *in vitro* for 3 days to generate Th1, Th2, Th17 or Treg cells. Glut family mRNA copy number was measured in naïve, stimulated or differentiated CD4 T cells from control (Ctrl) or knockout mice (KO). Data shown are mean \pm SD of pooled T cell mRNA from 2 mice. MC Rudolph and SM Anderson contributed to this data.

3.2.5 Glut1 is required for Teff expansion in colitis

Colitis is driven by Th1 and Th17 Teff and suppressed by Treg (Brand, 2009). The requirement of Teff for Glut1 to induce IBD was first tested using an adoptive transfer model (Mottet *et al.*, 2003) in which sorted naïve control and CD4CreGlut1^{fl/fl} T cells were transferred into immunodeficient Rag1^{-/-} recipients. The NSAID piroxicam was given two weeks after T cell transfer to induce gut damage and trigger disease. Importantly, CD4CreGlut1^{fl/fl} T cells were unable to effectively promote IBD as indicated by weight loss (**Fig. 3.6A**; day 0 indicates the start of piroxicam treatment), while control T cells induced significant weight loss over time. CD4CreGlut1^{fl/fl} T cells lacked Glut1 throughout the course of the experiment (**Fig. 3.6B**). Importantly, both total (**Fig. 3.6C**) and cytokine producing (**Fig. 3.6D, E**) T cell numbers were decreased by Glut1-deficiency.

To test if acute deletion of Glut1 also affected IBD, sorted naïve T cells or nTreg from control or UbiCreERT2Glut1^{fl/fl} mice were adoptively transferred individually or in combination into Rag1^{-/-} hosts. After two weeks to allow homeostatic expansion, mice were treated with piroxicam to trigger IBD followed by tamoxifen to activate CreERT2 and delete Glut1 in Teff or Treg subsets in vivo. Although Glut1 deletion was incomplete (**Fig. 3.7A**), UbiCreERT2Glut1^{fl/fl} T cells failed to induce inflammation, gut hyperplasia or granuloma, which were observed with control T cells (**Figs. 3.7B panels i and ii**). Broadly scoring colitis including architectural distortion, crypt abscesses,

severity of inflammation, ulceration, and percent of bowel affected, suggested mice that received Glut1-deficient T cells were resistant to severe colitis (**Fig. 3.7C**).

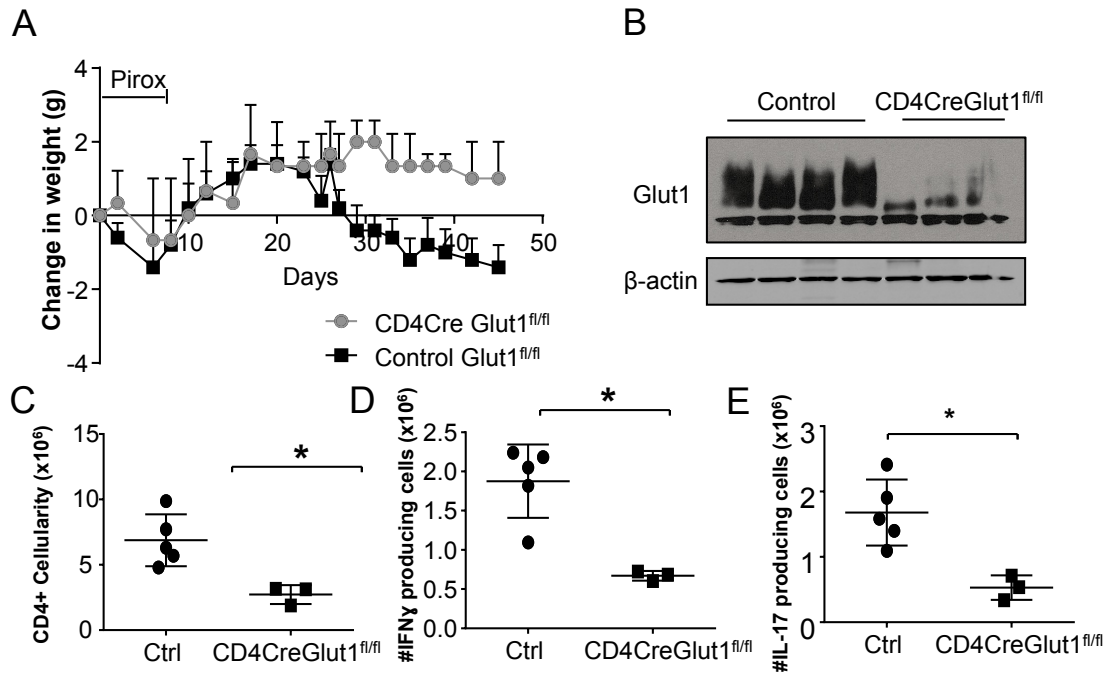


Figure 3.6: Glut1 is required for Teff expansion and function in colitis

Naïve control (Glut1^{fl/fl}) or CD4CreGlut1^{fl/fl} Teff were transferred into Rag1^{-/-} hosts and colitis was triggered 2 weeks later via piroxicam (Pirox; day 0 on start of Pirox) exposure and animal weights measured over time (A). (B) Glut1 expression by immunoblot of CD4 T cells isolated from the spleen and mesenteric lymph nodes of control (Glut1^{fl/fl}) or CD4CreGlut1^{fl/fl} T cells 4-5 weeks later. (C) The number of CD4 T cells or (D, E) IFNγ or IL-17 producing cells in the spleen was determined using flow cytometry.

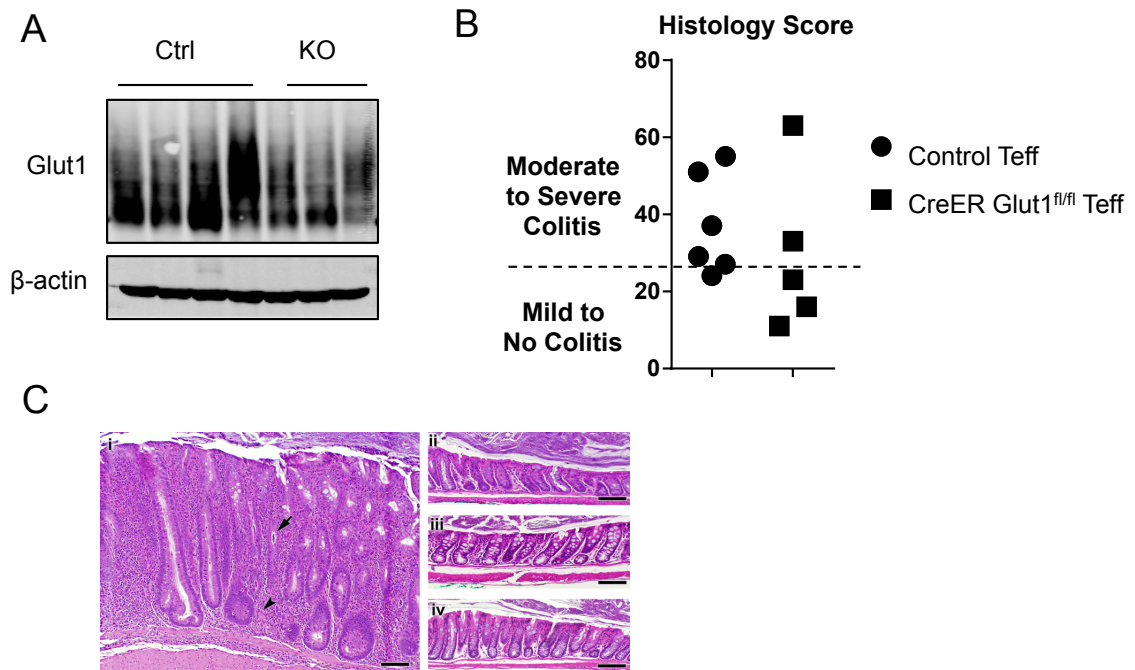


Figure 3.7: T cell expression of Glut1 is required for severe colitis

Rag1^{-/-} mice were reconstituted intraperitoneally via injection of naïve effector Rag1^{-/-} mice were injected with control (CreERGlut1^{+/+}, Glut1^{fl/fl}) or CreERGlut1^{fl/fl} naïve Teff. Colitis was triggered by piroxicam exposure 2 weeks after T cell transfer. Animals were then treated with tamoxifen to activate Cre. (A) CD4 T cells were isolated and Glut1 expression was examined by immunoblot. (B) H&E stained colon sections were examined by microscopy and assessed for colitis severity; a score above 22 indicates moderate to severe colitis. (C) H&E histology of proximal colon from mice that received (i) naïve control T cells, (ii) naïve CreERGlut1^{fl/fl} T cells, (iii) naïve control T cells plus control Treg, or (iv) naïve control T cells plus CreERGlut1^{fl/fl} Treg. Bar indicates 100µm; arrow indicates cryptic abscess and arrowhead indicates a granuloma. Data (A-C) are representative of three independent experiments. LP Hale: B,C.

3.2.6 Glut1 is required *in vivo* for Teff but not Treg expansion in colitis

UbiCreER^{T2}Glut1^{fl/fl} T cells failed to induce inflammation and were resistant to severe colitis (**Fig. 3.7**). Importantly, total numbers of Glut1-deficient CD4 T cells in the spleen and mesenteric lymph nodes were significantly reduced relative to control T cells 4 weeks after tamoxifen treatment (**Fig. 3.8A**). Glut1-deletion also reduced cytokine production by CD4 Teff, as fewer IFN γ and IL-17-producing cells were present in mice receiving Glut1-deficient T cells (**Fig. 3.8B, C**).

Importantly, Glut1-deficient Treg remained functional *in vivo* and capable of inhibiting Teff in IBD. Glut1-deficient nTreg suppressed Teff expansion similar to wild type nTreg, as total CD4⁺ cell numbers in the spleen and mesenteric lymph nodes and gut pathology were identical when control or Glut1-deficient nTreg were transferred together with control Teff cells (**Fig. 3.7C panels iii and iv, 8D**). Thus, while Teff populations require Glut1 expression to drive inflammatory colitis, Treg can suppress Teff-mediated inflammatory Teff expansion irrespective of Glut1 expression. Dependence of activated T cells and Teff on Glut1 may, therefore, allow selective targeting of T cell glucose metabolism to suppress inflammatory responses and promote tolerance and immune suppression.

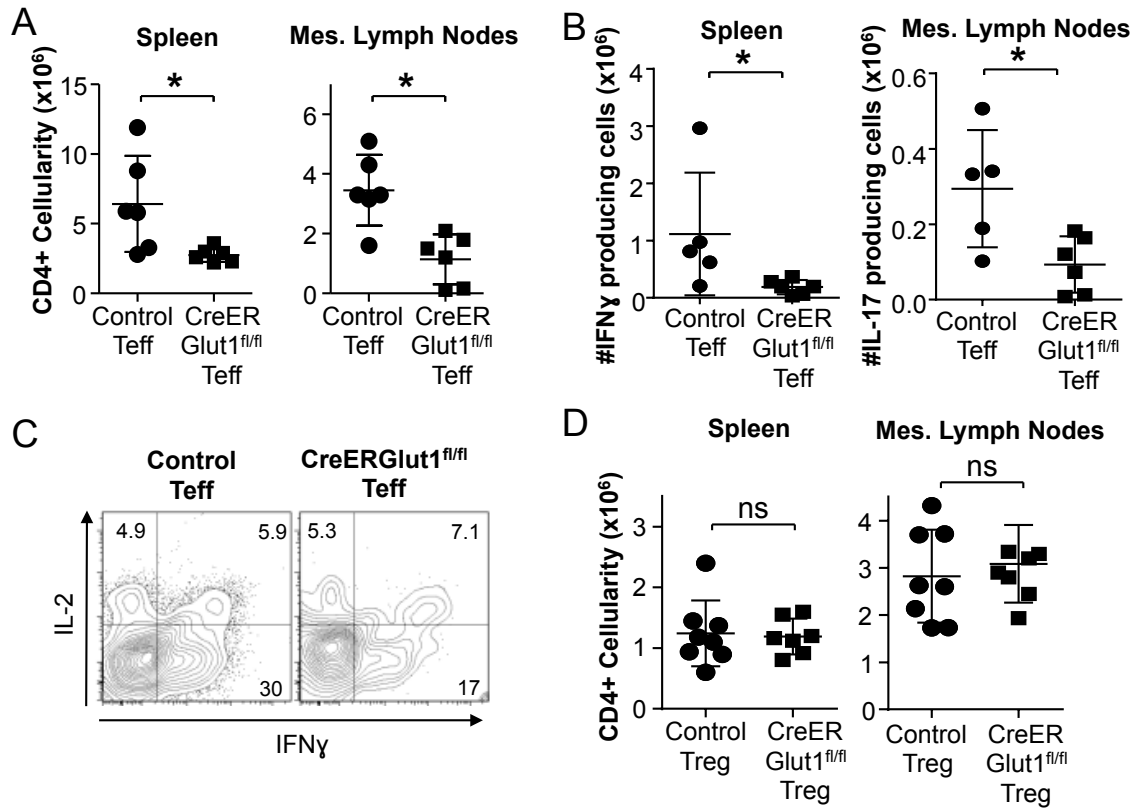


Figure 3.8: Glut1 is acutely required for Teff, but not Treg, expansion and function in colitis

Rag1^{-/-} mice were injected with control (CreERGlut1^{+/+}, Glut1^{fl/fl}) or CreERGlut1^{fl/fl} naïve Teff. Colitis was triggered by piroxicam exposure 2 weeks after T cell transfer. Animals were then treated with tamoxifen to activate Cre. (A) The number of CD4 or (B, C) IFN γ or IL-17 producing T cells in the spleen and mesenteric (mes) lymph nodes was determined after 4 weeks by flow cytometry. (D) Rag1^{-/-} mice were co-injected with wild type naïve Teff and either control (CreERGlut1^{+/+}, Glut1^{fl/fl}) or CreERGlut1^{fl/fl} nTreg. Mice were treated after 2 weeks with piroxicam and tamoxifen to trigger IBD and activate Cre and CD4 T cells were determined after 4 weeks. Data are representative of 3 independent experiments.

3.3 Discussion

The *in vivo* metabolic demands of T cell activation, proliferation, and differentiation and the programs that T cells initiate to support these needs may provide new targets to modulate the immune response (MacIver *et al.*, 2013). *In vivo*, T cells require mitochondrial ROS (Sena *et al.*, 2013), lipid synthesis (Kidani *et al.*, 2013), and amino acid uptake (Sinclair *et al.*, 2013). The mechanism and role of glucose uptake in T cell homeostasis, activation, and differentiation have not been directly tested *in vivo*. Here, our approach that directly targeted the first step of glucose metabolism by genetic deletion of Glut1 identified a selective reliance on this glucose transporter in T cell proliferation and CD4 Teff expansion to induce GvHD and colitis, while Treg are Glut1-independent.

In addition to the cMyc (Wang *et al.*, 2011) and Estrogen Related Receptor α (ERR α) (Michalek *et al.*, 2011a) transcription factors that regulate T cell metabolism, mTOR and the PI3K-Akt-mTOR complex 1 (mTORC1) signaling pathway can promote Glut1 cell surface trafficking, glycolysis, and lipid synthesis (Duvel *et al.*, 2010; Wieman *et al.*, 2007) and Teff (Waickman and Powell, 2012). T cell-specific deletion of mTOR kinase to eliminate both mTORC1 and mTORC2 prevented generation of Teff, but allowed establishment of functional Treg (Delgoffe *et al.*, 2009). Conversely, specific deletion of mTORC1 activation or components has been shown in distinct settings to not

affect (Delgoffe *et al.*, 2011) or to prevent (Zeng *et al.*, 2013) Treg suppressive function. These data suggest that mTOR-driven glucose or lipid metabolism may be critical for Treg (Zeng *et al.*, 2013). However, decreased mTORC1 signaling can lead to exacerbated mTORC2 activity (Zeng *et al.*, 2013) that can also promote glycolytic metabolism (Gubser *et al.*, 2013; Masui *et al.*, 2013) and may thus suppress Treg activity. Indeed simultaneous deletion of essential mTORC1 and mTORC2 components or mTOR itself restored Treg function (Delgoffe *et al.*, 2009; Zeng *et al.*, 2013). This complex interplay between the multiple mTOR-induced signaling and metabolic events has hindered mechanistic interpretation of the specific role of metabolic regulation in T cell function and fate. Our studies directly test the role of Glut1 upregulation in T cell activation and subsets to show a selective Glut1-dependence of activated Teff and Glut1-independence of Treg.

Glucose uptake is mediated through the fourteen-member Glut transporter family, of which we show CD4 T cells express Gluts1, 3, 6, and 8. Each was regulated in T cell activation and differentiation, with highest expression of Glut1 and Glut3. Gluts 6 and 8 were expressed at relatively low levels and their specific roles are unclear at this time. The high Glut3 expression and Glut1-independence of resting T cells and CD8 Teff suggests a role for Glut3-mediated glucose uptake. While CD4 Teff cells were reliant on Glut1-directed metabolism, functional Treg could be generated from naïve T cells lacking Glut1, despite expressing only low levels of Glut3. These data are consistent with Treg use of mitochondrial oxidative pathways rather than glucose metabolism

(Michalek *et al.*, 2011b; Shi *et al.*, 2011). In support of an alternate metabolic program, Treg can be generated in the absence of glucose (Michalek *et al.*, 2011b) or in the presence of 2DG (Shi *et al.*, 2011).

Despite simultaneous expression of multiple glucose transporters, our data demonstrate a specific requirement for Glut1 in both activated mouse and human T cells *in vitro* and *in vivo*. The Glut1-dependent molecular switch to elevate glycolysis was critical for rapid human T cell growth and proliferation, as Glut1 knockdown suppressed glycolysis and slowed the transition of human T cells from quiescence to proliferation. Likewise, activation of murine cells led to a dependence on Glut1 to support cell growth, proliferation, and prevention of apoptosis. Thymocytes in the proliferative DN3-DN4 transition expressed Glut1 at a high level and were also sensitive to Glut1-deficiency. In the absence of Glut1, activated mature T cells failed to increase glucose uptake and glycolysis beyond resting levels, had selectively reduced growth and proliferation, and many cells underwent apoptosis. These broad inhibitory effects of Glut1-deficiency were potentially due to AMPK activation and suppression of mTORC1 *in vitro* and *in vivo* that resulted in fewer inflammatory cytokine-producing cells.

The differential requirements of specific T cell populations for Glut1 may reflect specific functional needs for a highly glycolytic metabolism. Aerobic glycolysis is closely linked with cell growth to generate increased mass for cell proliferation (Vander Heiden *et al.*, 2009) and Glut1-deficient T cells failed to grow after stimulation.

Proliferation was also suppressed and cell death increased, possibly as a consequence of inadequate nutrients to support biosynthesis and prevent AMPK suppression of mTORC1. In addition, Glut1-deficient CD4 Teff cells also had reduced production of IFN γ . Glucose-deprivation has been shown to lead to a specific reduction of IFN γ production *in vitro* (Cham and Gajewski, 2005; Jacobs *et al.*, 2008) and glycolytic flux has been implicated in IFN γ translation (Chang *et al.*, 2013). Indeed, we found both reduced cell numbers and decreased inflammatory cytokine production by Glut1-deficient Th1 and Th17. In contrast to Teff, our data show that Treg do not require Glut1 and appear to utilize an alternate metabolic program *in vitro* and *in vivo*. It remains to be determined, however, if Treg induce aerobic glycolysis using an alternate glucose transporter or if they proliferate using a distinct metabolic program. The metabolic requirements of different T cell activation states and subsets may also play a broad role in immune homeostasis or disease, supporting specific T cell populations in distinct tissues and immunologic settings.

Identifying biochemical requirements for T cell activation and the generation of effector and regulatory T cells has been a long sought goal in efforts to treat inflammatory diseases. Mechanisms that control cell metabolism to support the specific functional needs of these cells have been described only recently (MacIver *et al.*, 2013), but have proven promising (Bian *et al.*, 2009; Eleftheriadis *et al.*, 2013; Ostroukhova *et al.*, 2012; Shi *et al.*, 2011). To date, pharmacologic approaches have provided limited

mechanistic insight, and the role of glucose uptake has been uncertain. Data presented here demonstrate that despite expression of multiple Glut family transporters, Glut1 is specifically required for the cell-intrinsic metabolic program of activated T cells and CD4 Teff *in vitro* and *in vivo* to drive inflammation in both colitis and GvHD. These data show that despite a potential requirement for lipid oxidation (Byersdorfer *et al.*, 2013; Gatza *et al.*, 2011), Glut1 is central in the metabolism of Teff and in GvHD. Glut1, however, is not required in all settings, as resting T cells, CD8 Teff, and Treg were capable of Glut1-independent function. Collectively, these findings demonstrate that distinct T cell subsets utilize selective metabolic programs with differing dependence on Glut1. Understanding the roles and regulations of specific nutrient transporters in T cell activation and subsets may now provide new opportunities to exploit metabolic distinctions of cells in the immune system to control inflammatory diseases.

4. Intrinsic Metabolic Programming and Pyruvate Dehydrogenase Kinase Control CD4 T cell Differentiation and Autoimmunity

4.1 Introduction

CD4 T Lymphocytes play key roles to mediate or suppress immunity and inflammatory or autoimmune diseases. Antigen exposure initially leads to T cell activation to induce rapid growth and proliferation. Depending on the cytokine environment during activation, CD4 T cells are then stimulated to differentiate into effector (Teff; Th1, Th2 and Th17) or regulatory (Treg) subsets. Each of these subsets plays a unique role in the adaptive immune system, with Teff driving immunity and inflammation while Treg play an opposing role, suppressing Teff to limit excessive inflammatory responses (Zhu *et al.*, 2010). The balance between Teff and Treg is crucial to provide sufficient immune protection without promoting autoimmunity. Indeed, many autoimmune diseases, including multiple sclerosis and inflammatory bowel disease (IBD) involve an imbalance of Teff to Treg or decreased Treg function (Buckner, 2010). Th17, in particular, play a key pro-inflammatory role in many autoimmune diseases including experimental autoimmune encephalomyelitis (EAE), IBD and Graft-vs-Host Disease (Cua *et al.*, 2003; Yen *et al.*, 2006; Serody and Hill, 2012). Therefore, identifying characteristics of each T cell population to allow the balance of Teff and Treg to be modulated may provide a way to prevent or suppress autoimmunity.

Emerging evidence has suggested that CD4 T cell metabolism may allow targeting of select T cell populations. The transition from a naïve to activated lymphocyte requires rapid growth and proliferation. With these requirements comes a demand for substantial metabolic reprogramming to provide the biosynthetic precursors and energy necessary for effector function (Fox *et al.*, 2005; Vander Heiden *et al.*, 2009). This reprogramming involves decreased lipid oxidation and an increase in glucose uptake and glycolysis as well as amino acid transport and glutaminolysis (Frauwirth *et al.*, 2002; Jacobs *et al.*, 2008; Maciver *et al.*, 2008; Sinclair *et al.*, 2013; Wang *et al.*, 2011). Importantly, metabolic reprogramming of Teff and Treg leads to distinct metabolic programs for each subset.

While Teff require large amounts of glucose and a high rate of glycolysis to support their energetic needs, Treg are able to expand and function in the absence of glucose (Michalek *et al.*, 2011a; Shi *et al.*, 2011). Treatment of activated T cells with the glycolytic inhibitor 2-deoxyglucose (2DG) leads to Treg accumulation and inhibition of IL-17 production (Shi *et al.*, 2011). In addition, we have shown that exogenous fatty acids suppress Teff generation while promoting Treg and that Treg have high rates of lipid oxidation *in vitro* (Michalek *et al.*, 2011a). The role of specific metabolic programs and substrate usage for activation and differentiation of Teff and Treg, however, is uncertain.

One bifurcation point in glucose metabolism that may play a central role in the glycolytic or oxidative roles of Teff and Treg is determination of the fate of glycolytic pyruvate through Pyruvate Dehydrogenase (PDH). PDH mediates the conversion of cytosolic pyruvate into mitochondrial acetyl-CoA for oxidative metabolism. PDH is inhibited by Pyruvate Dehydrogenase Kinase (PDHK) to suppress pyruvate oxidation and instead promote conversion to lactate and high rates of glycolysis. PDHK has four isoforms that are regulated by oncogenic signaling and hypoxia (Hitosugi *et al.*, 2011; Korotchkina and Patel, 2001). Indeed, cancer cells are also highly glycolytic and PDHK inhibition with dichloroacetate (DCA) can suppress glycolysis and lead to increased oxidative metabolism and reactive oxygen species production (Michelakis *et al.*, 2010). DCA has been shown to reduce pro-inflammatory cytokine production and promote FoxP3 expression *in vitro* and in several *in vivo* inflammatory models (Bian *et al.*, 2009; Eleftheriadis *et al.*, 2013; Ostroukhova *et al.*, 2011). Importantly, these studies examined the effect of DCA in the context of total PBMCs or mouse models and therefore the direct effect on T cells is unknown. The mechanism of DCA action and how Teff are selectively suppressed while Treg are promoted has not been established.

Here, we examine the metabolic programs underlying each T cell subset to identify distinct metabolic pathways that will allow selective targeting of different T cell populations. We show that Treg oxidize not only lipids at a high rate, but also glycolytic pyruvate. Gene expression and metabolomics data suggested PDHK isoform 1

(PDHK1) as a modulator of Th17 and Treg differentiation and survival. Indeed, PDHK1-deficiency or inhibition with DCA led to ROS stress selectively in Th17 cells that inhibited their survival and proliferation. As a consequence, DCA was capable of suppressing CD4 T cell mediated inflammation in both inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE) models. Together, these metabolic data have identified PDH as a key regulatory point in CD4 T cell subset fate and show that inhibition of PDHK1 leads to selective ROS generation and targeting of Th17 cells *in vivo* in inflammatory and autoimmune disorders.

4.2 Results

4.2.1 Teff and Treg utilize different metabolic pathways and have distinct fuel capacities

Previous studies have identified basic metabolic differences between effector T cells (Teff; Th1, Th2 and Th17) and regulatory T cells (Treg). However, underlying distinctions that drive the CD4 T cell subsets and may allow selective targeting of inflammatory subsets are poorly defined. To examine the metabolic phenotype of the CD4 T cell subsets, Teff and Treg were differentiated *in vitro* and oxygen consumption and lactate production were measured using an extracellular metabolic flux analyzer. Cells were cultured in the absence of glucose and the rate of extracellular acidification (ECAR), a measurement of lactate production, was determined upon re-addition of glucose (**Fig 4.1a**). All CD4 T cell subsets increased ECAR, although Th1 and Treg

increased less than Th2 and Th17 cells. Oligomycin was then added to block mitochondrial ATP production and promote maximal rates of glycolysis in compensation. Importantly, Th1, Th2, and Th17 cells each showed a robust increase in ECAR following oligomycin treatment, but Treg were largely unchanged. These data indicate that Treg were performing glycolysis at maximal rates following glucose addition and have limited capacity to increase this pathway. Teff, in contrast, generate glycolytic lactate at a high rate and can further elevate glycolytic rate when required to generate ATP.

The glycolytic capacity and glycolytic reserve were both severely impaired in Treg compared to Teff (**Fig 4.1b, c**). Therefore, when glucose is the only fuel available, Teff efficiently perform glycolysis while Treg are unable to increase their glycolytic capacity.

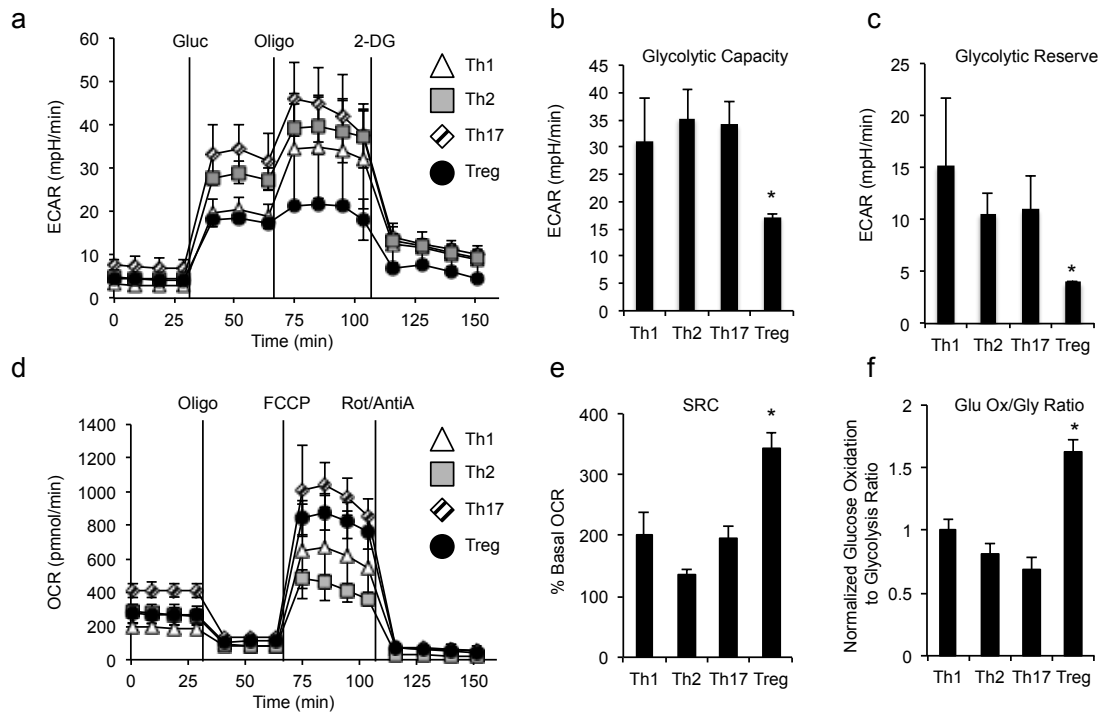


Figure 4.1: T_{eff} and T_{reg} utilize different metabolic pathways and have distinct fuel capacities

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells. (a-c) The rate of extracellular acidification (ECAR) was assessed after the addition of 10mM glucose (gluc) and in response to the metabolic inhibitors oligomycin (oligo) and 2-deoxyglucose (2-DG). Shown are the time course (a), and calculations of glycolytic capacity (b) and glycolytic reserve (c). (d, e) Oxygen consumption (OCR) was assessed basally and in response to the mitochondrial inhibitors oligomycin (oligo), FCCP, rotenone and antimycin A (Rot/AntiA). Shown are the time course (d), and calculation of spare respiratory capacity (e). (f) Glucose oxidation was measured in the T cell subsets and the ratio of glucose oxidation to glycolysis is graphed. Data is shown as mean \pm SD (b, c, e, f) and all data are representative of at least 3 independent experiments.

Mitochondrial and oxidative metabolism can play a key role to support T cell activation and proliferation (Chang *et al.*, 2013; Sena *et al.*, 2013). While Treg have been shown to have high rates of lipid oxidation, mitochondrial oxidation of glycolysis-derived pyruvate had not been examined. Mitochondrial oxygen consumption rate (OCR) was therefore measured in each CD4 subset in glucose containing media. Prior to addition of metabolic inhibitors, Treg had an intermediate level of oxygen consumption relative to Teff, with Th17 cells maintaining the highest basal rate of oxygen consumption (**Fig 4.1d**). Oligomycin treatment to inhibit mitochondrial ATP production suppressed oxygen consumption in each subset to an equivalently low level, indicating that oxygen consumption was tightly coupled to ATP generation for all T cell subsets. Upon the addition of the protonophore FCCP to uncouple oxidative phosphorylation from electron transport and allow maximal respiration, Treg and Th17 greatly upregulated oxygen consumption. When compared to the basal rates of oxygen consumption, these data show that Treg have the greatest spare respiratory capacity (SRC) of the subsets (**Fig 4.1e**). Thus, Treg have low glycolytic production of lactate and cannot further elevate this pathway, yet have the greatest capacity for mitochondrial oxidation when given glucose as a fuel.

These data suggest that while all CD4 T cell subsets utilize glucose, Teff preferentially convert pyruvate to lactate while Treg oxidize this fuel in the mitochondria. To directly measure the fate of glycolysis-derived pyruvate, CD4 T cell

subsets were provided radiolabeled glucose and glycolytic flux and glucose oxidation were measured. The ratio of glucose oxidation to glycolysis was found to be higher in Treg, supporting the conclusion that this subset preferentially oxidizes glucose rather than converting pyruvate to lactate (**Fig 4.1f**). Overall, these data indicate that Treg utilize mitochondrial oxidative pathways using both lipids (Michalek *et al.*, 2011a) and glucose as fuels while Teff have low levels of mitochondrial oxidative metabolism either fuel and primarily perform aerobic glycolysis to lactate.

4.2.2 Inhibition of glycolysis or mitochondrial oxidation selectively impacts Teff or Treg survival, proliferation and function

Although flux analysis revealed that Teff and Treg utilize distinct glycolytic or mitochondrial metabolic programs, the reliance of these subsets on each pathway was not clear. To determine if each CD4 subset required these programs, CD4 T cell subsets were labeled with the proliferation indicator dye Cell Trace Violet (CTV) and differentiated *in vitro* and treated with either low dose 2-deoxyglucose (2DG) or rotenone, to inhibit glycolysis or electron transport, respectively. 2DG treatment inhibited the proliferation of each Teff subset but had a minimal effect on Treg (**Fig 4.2a**). Conversely, rotenone did not affect Teff, but sharply reduced Treg proliferation.

Differentiation of each CD4 T cell subset is characterized by induction of specific transcription factors as cells undergo DNA replication and division (Zhu *et al.*, 2010). Given the reliance of Teff on glycolysis and Treg on mitochondrial metabolism for

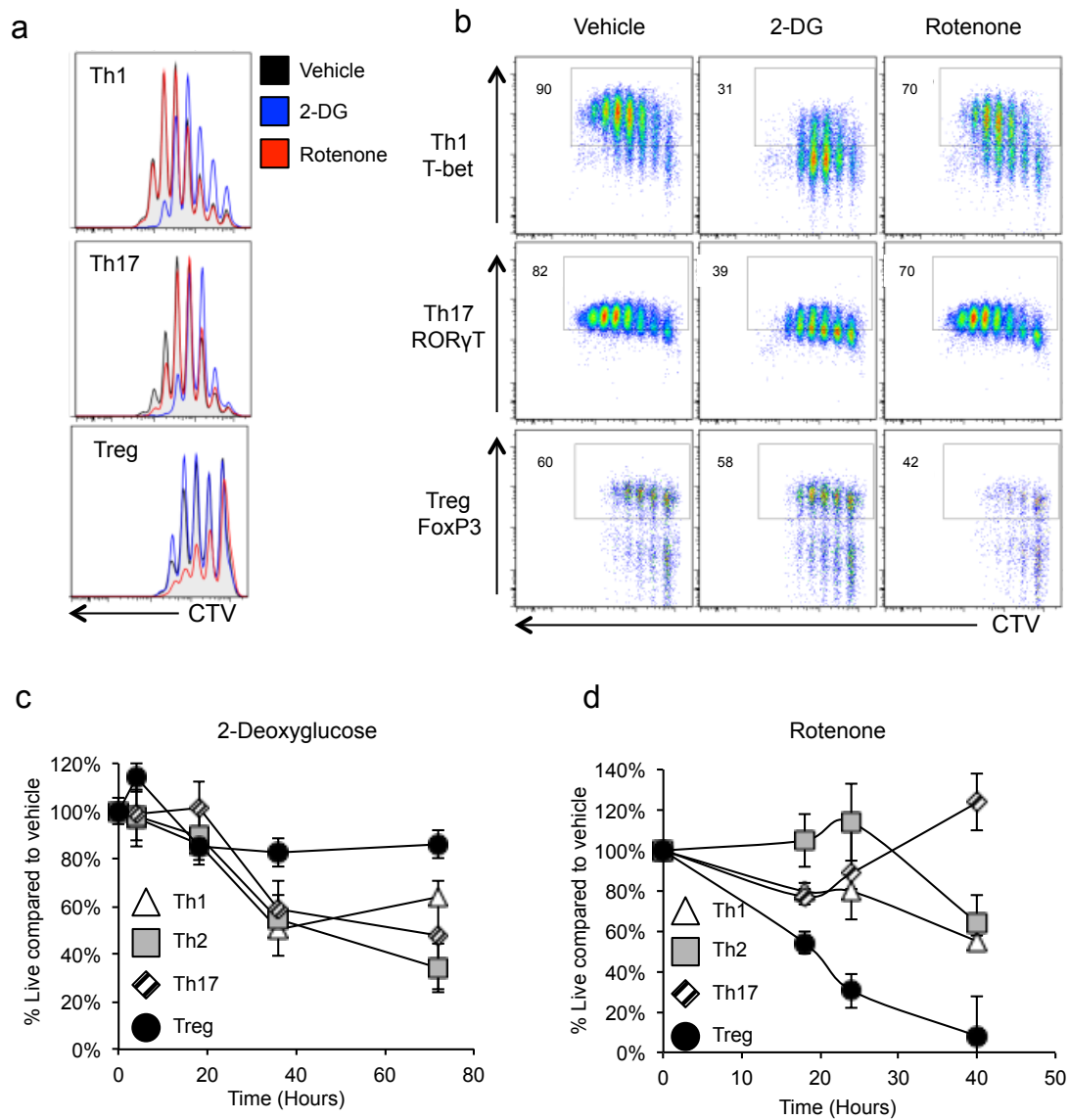


Figure 4.2: Inhibition of glycolysis or mitochondrial oxidation selectively impacts T cell survival, proliferation and function

(a, b) CD4⁺CD25⁻ T cells were labeled with CellTrace Violet (CTV) and polarized *in vitro* for 3 days to generate Th1, Th2, Th17 or Treg cells. Cells were treated with 250μM 2-deoxyglucose (2-DG) or 5nM rotenone and (a) proliferation or (b) transcription factor staining was assessed by CTV dilution after 72 hours. (c, d) CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days and then incubated with (c) 250μM 2-DG or (d) 25nM rotenone and survival was determined by propidium iodide exclusion. Data is shown as mean ± SD (c, d) and all data are representative of at least 3 independent experiments.

proliferation, it was possible that the ability of subsets to express these transcription factors was also metabolically sensitive. Consistent with this notion, 2DG treatment suppressed induction of T-bet in cells cultured in Th1 conditions as well as ROR γ t in T cells cultured in Th17 conditions (**Fig 4.2b**). Importantly, T-bet and ROR γ t expression was inhibited at each equivalent cell cycle division. Likewise, expression of the T-bet and ROR γ t transcriptional targets, IFN γ and IL-17, respectively, was also suppressed by 2DG at each cell cycle (**Fig 4.3**). Induction of the Treg transcription factor FoxP3, however, was not affected by 2DG. The electron transport inhibitor, rotenone, had the opposite effect and only minimally suppressed T-bet and ROR γ t expression of Th1 and Th17 cells, and selectively reduced FoxP3 expression in Treg at each equivalent cell division.

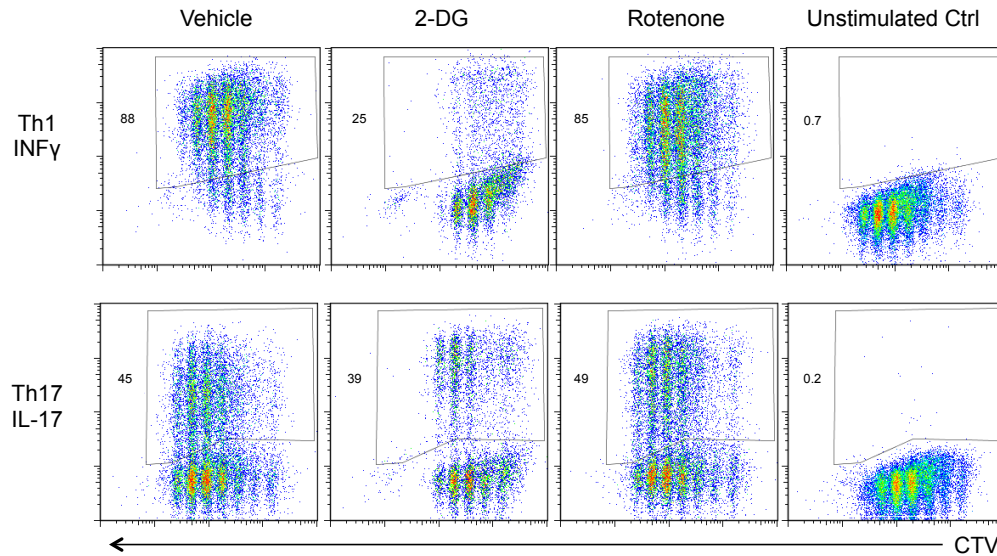


Figure 4.3: Inhibition of metabolic pathways selectively impacts T cell function

CD4⁺CD25⁻ T cells were labeled with CellTrace Violet (CTV) and polarized *in vitro* for 3 days to generate Th1, Th2, Th17 or Treg cells. Cells were treated with 250μM 2-deoxyglucose (2-DG) or 5nM rotenone and proliferation was assessed by CTV dilution and intracellular cytokine staining staining for IFNγ (Th1) or IL-17 (Th17) was performed after 72 hours. Data is representative of 3 independent experiments.

CD4 subsets may rely on specific metabolic programs not only for proliferation and differentiation, but also for survival. Teff and Treg subsets were therefore established *in vitro* and treated with 2DG or rotenone. Treg survived well in the presence of 2DG, only slightly affected by the glycolytic inhibition (**Fig 4.2c**). In contrast, Th1, Th2 and Th17 were sensitive to 2DG treatment and showed increased cell death (**Fig 4.2c**, **4.4a**). Teff were, however, relatively insensitive to rotenone, with the Th17 surviving better than in the vehicle condition (**Fig 4.2d**). Treg were extremely sensitive to rotenone

treatment, with less than 10% surviving after 40 hours of treatment (**Fig 4.2d, 4.4b**).

Overall, this data suggests that metabolism is fundamentally linked to CD4 T cell survival, proliferation and differentiation.

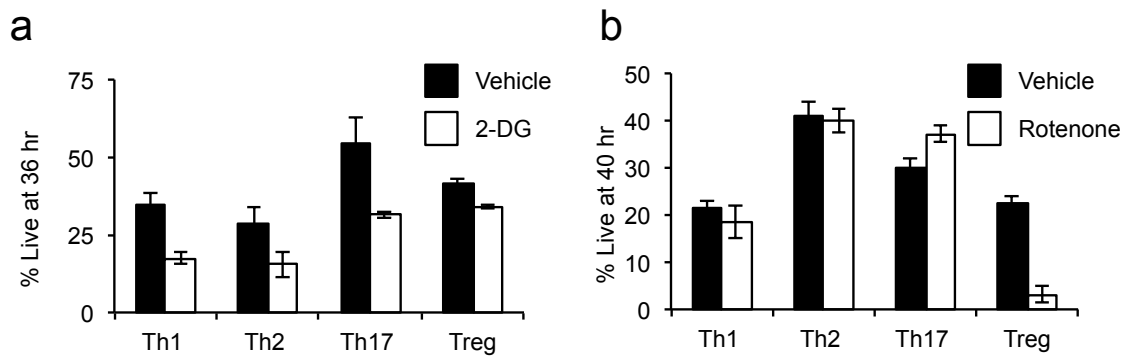


Figure 4.4: Inhibition of glycolytic or oxidative metabolic pathways selectively impacts Teff or Treg survival

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days and then incubated with (a) 250 μ M 2-DG or (b) 5nM rotenone and survival was determined by propidium iodide exclusion. Data is shown as mean \pm SD and data are representative of 3 independent experiments.

4.2.3 The CD4 T cell subsets have distinct metabolic phenotypes

The metabolic dependencies of CD4 T cell subsets may allow targeting of specific T cell populations. Therefore, we examined metabolite levels and metabolic gene expression of Teff and Treg in detail. Steady state metabolite levels were measured by GC/MS and LC/MS and unsupervised clustering analysis showed that each CD4 subset was metabolically distinct (**Fig 4.5**).

Importantly, Treg were the most disparate and were distinct from each Teff population, which were more similarly related. Pathway and principle component analyses demonstrated that glycolytic and mitochondrial metabolic pathways in particular were sharply different between Teff and Treg populations. Treg had lower levels of many of the glycolytic metabolites, including glucose-6-phosphate (G6P) and fructose-1,6-bisphosphate (FBP), with the largest differences between Th17 and Treg (**Fig 4.6a**). Treg, however, had a higher level of the starting materials glucose and fructose compared to Teff. These data suggest that Treg are capable of hexose uptake but do not consume these fuels to the same degree as Teff. TCA cycle metabolites were variable and no patterns were evident (**Fig 4.6b**).

We next examined expression of metabolic genes and proteins in each CD4 subset. CD4 T cells express several glucose transporters, including Glut1 and Glut3. The protein expression of Glut1 protein was elevated in Teff compared to Treg and Treg did not express detectable levels of Glut3 (**Fig 4.7a**). After uptake, glucose is

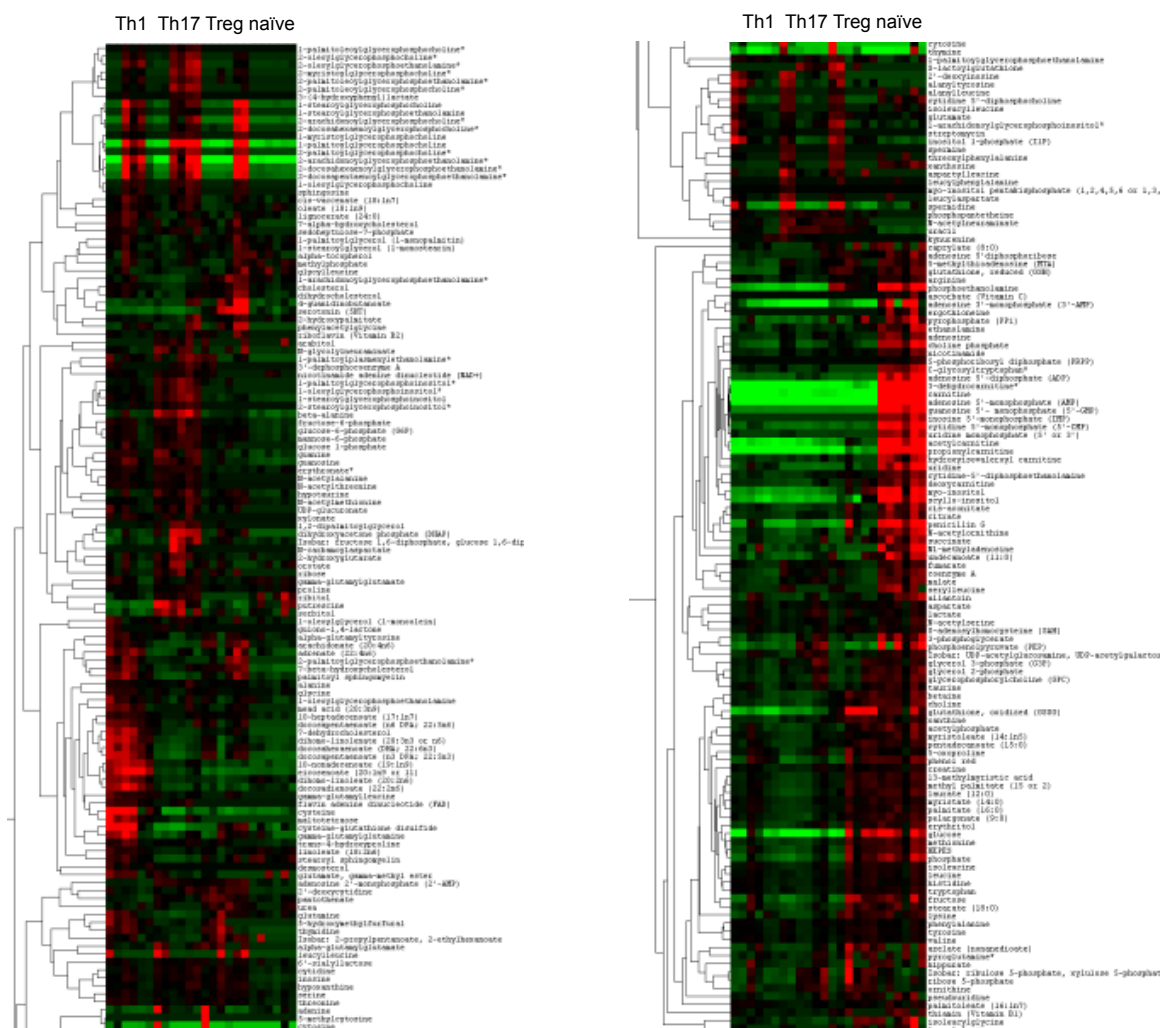


Figure 4.5: Metabolomic profiling of Teff and Treg show diverse metabolic pathway utilization

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells. T cell subset lysates were extracted and GC/MS and LC/MS/MS were performed for determination of cellular metabolites. Heat map showing relative levels of each metabolite and unsupervised hierarchical clustering. JA Chi contributed to this data.

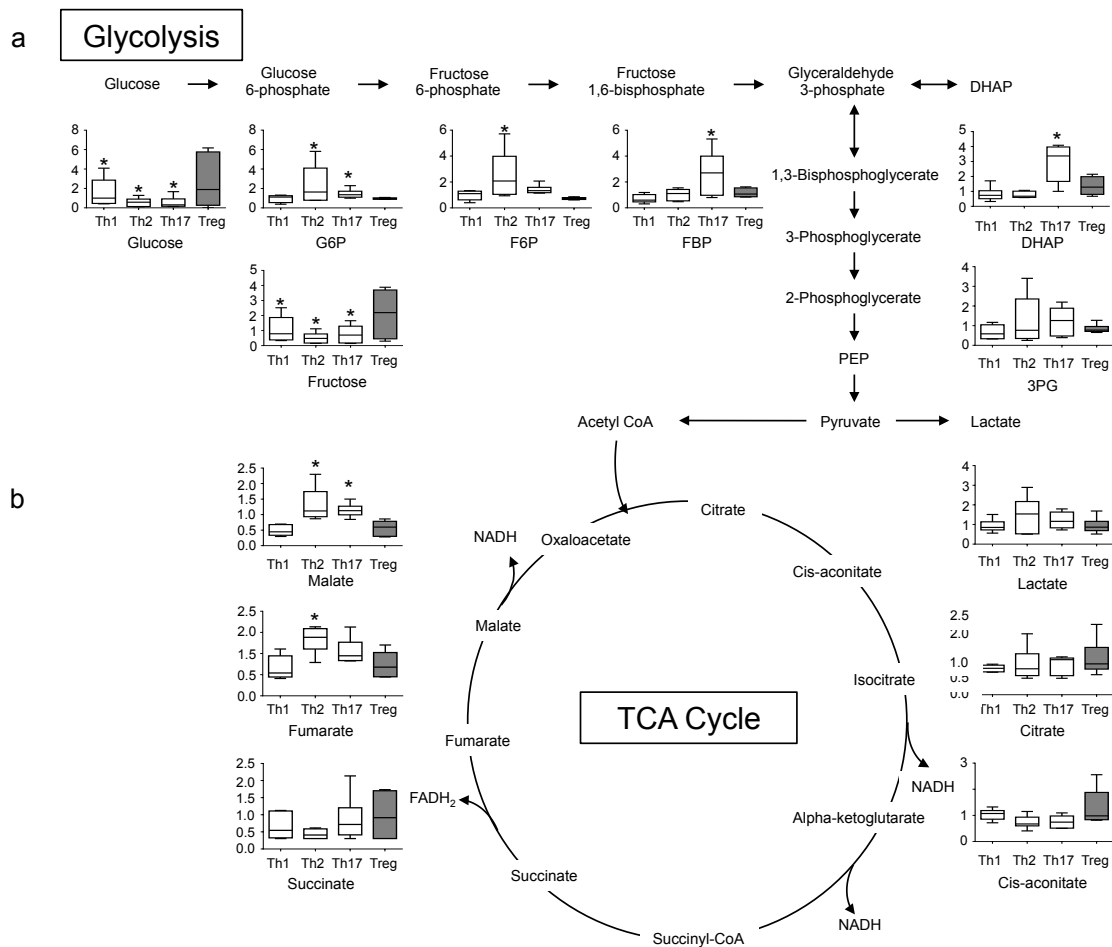


Figure 4.6: Metabolomic profiling of the T cell subsets shows diverse metabolic profiles

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells. T cell subset lysates were extracted and split into equal parts for analysis on GC/MS and LC/MS/MS platforms for determination of cellular metabolites. Relative levels of each metabolite in the glycolysis and TCA cycle pathways are shown.

phosphorylated by hexokinase (HK), of which there are four isoforms. Surprisingly, Treg expressed primarily HK1 while the Teff subsets mainly express HK2 and HK3 (**Fig 4.7a**). Glycolytic metabolic genes were then examined and, interestingly, CD4 T cells express a single isoform of each glycolytic gene (**Fig 4.7b**). While generally elevated in the differentiated cells compared to naïve T cells, Teff largely have higher levels of expression of the glycolytic genes compared to Treg, with the comparison being most striking between Th17 and Treg (**Fig 4.7b**). Of the effector T cells, Th17 had the highest expression of glycolytic genes, followed by Th2 and finally Th1.

Mitochondrial gene and protein expression was then examined in the CD4 T cell subsets. Treg had slightly elevated levels of many of the electron transport chain proteins as well as higher levels of CPT1A and cytochrome C compared to Teff, although each subset was unique (**Fig 4.7c**). The expression of the PDH genes Pdh1a and Pdhb were particularly interesting given their role to direct pyruvate to acetyl-CoA and the TCA cycle and elevated expression in Th17 and Treg compared to naïve, Th1 and Th2 subsets (**Fig 4.7d, top left panel**). The expression of genes involved in the TCA cycle and electron transport were similar between all of the CD4 T cell subsets, suggesting that while Teff preferentially use glycolysis, both Teff and Treg can utilize their mitochondria for energy production (**Fig 4.7d, Fig 4.8**).

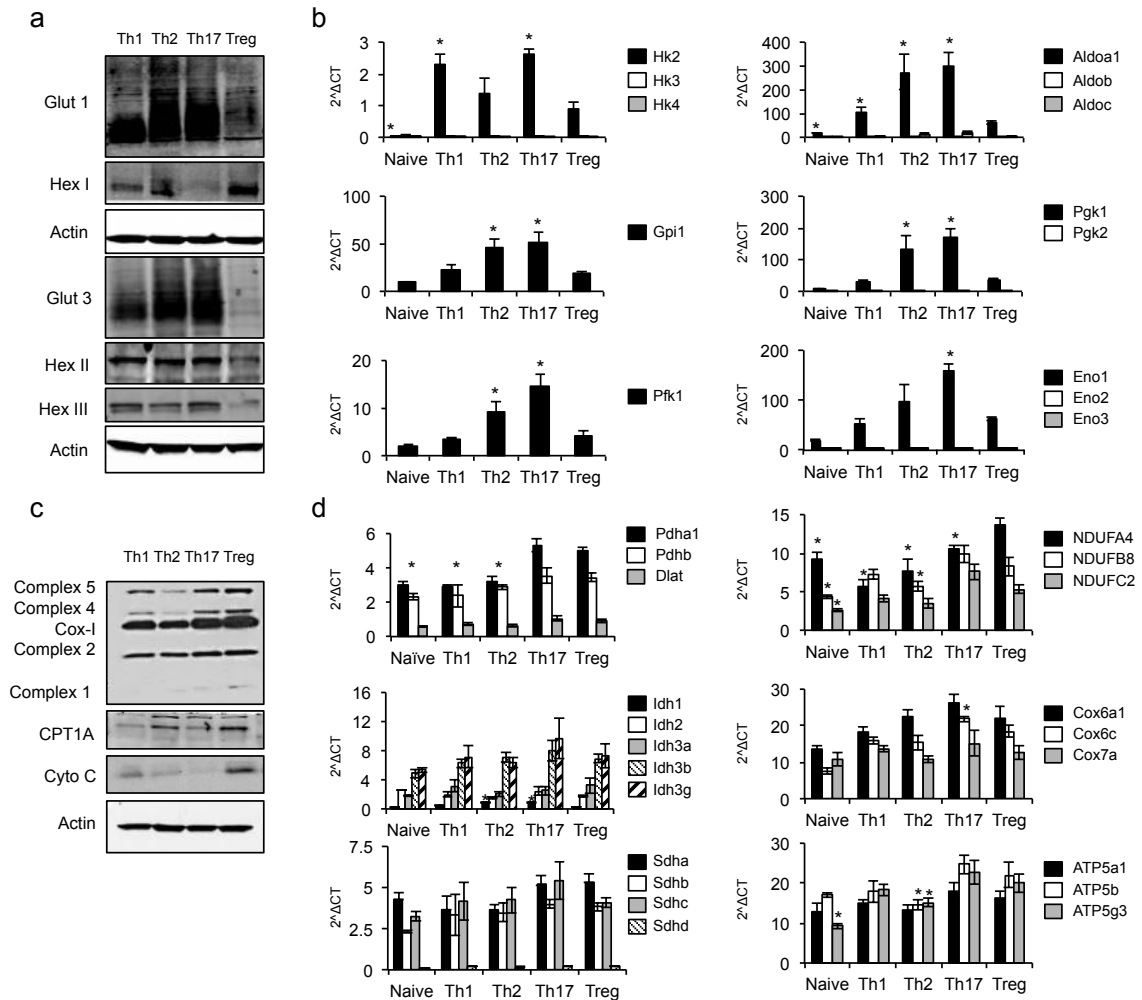


Figure 4.7: CD4 T cell subsets have distinct metabolic gene and protein expression

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells for (a, c) immunoblot or (b, d) real-time PCR. (b, d) Data shown is mean \pm SD of 3 biological replicates and shown as $2^{\Delta\Delta CT}$ normalized to the geometric mean of TBP and BGU. Data are representative of 3 (a, c) or 2 (b, d) independent experiments.

Mitochondrial metabolism

Glucose metabolism

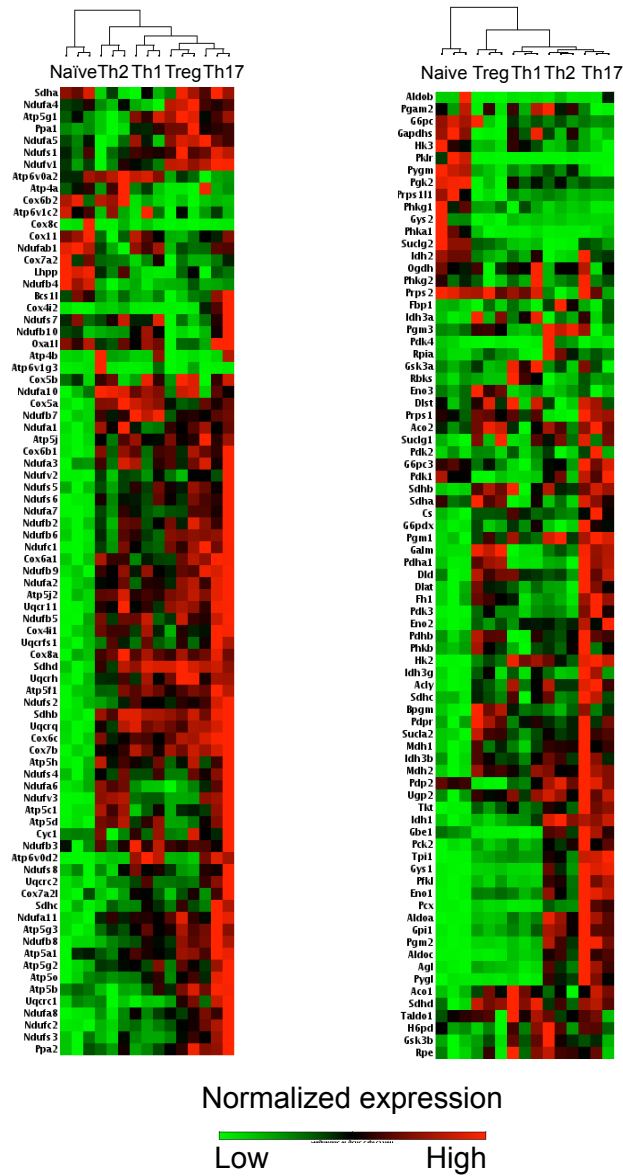


Figure 4.8: CD4 T cell subsets have distinct metabolic gene expression profiles

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells for real-time PCR of mitochondrial metabolism and glucose metabolism genes. Data are representative of 2 independent experiments.

Besides glucose utilization, T cells can also oxidize amino acids and lipids. Therefore, the amino acid and acyl carnitine profiles of the T cell subsets were examined to determine if subsets differed in fuel usage. All four of the subsets had higher intracellular amino acid levels relative to naïve T cells (**Fig 4.9a**). Within the individual T cell subsets, levels of the amino acids were variable. Acyl-carnitines, which reflect mitochondrial fuels derived from cellular lipids and amino acids, were also quite variable between the CD4 T cell subsets. However, naïve, Th2 and Treg had increased C2 and C4-OH levels that indicate acetyl CoA and ketone body species, respectively (**Fig 4.9b, c**). C4-OH (hydroxybutylcarnitine) is elevated in cells undergoing lipid oxidation (Schooneman *et al.*, 2013) and these findings suggest that Th2 and Treg utilize fatty acids to a greater extent than Th1 and Th17. Together, these metabolic data suggest that each CD4 T cell subset has a unique metabolic phenotype, yet point to specific metabolic distinctions in fuel consumption patterns and dependencies between Treg and the Teff subsets.

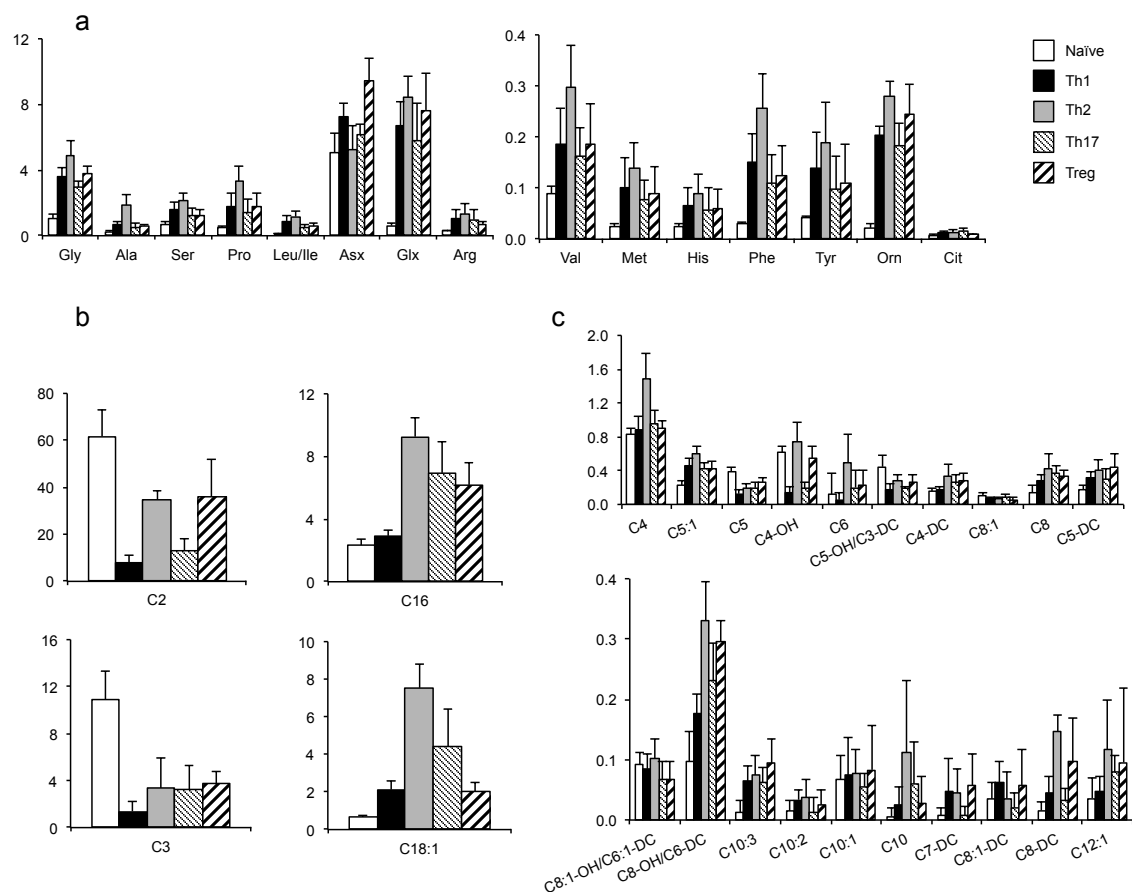


Figure 4.9: CD4 T cell subsets have different acyl carnitine and amino acid profiles

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells. T cell subset lysates were extracted and GC/MS was performed to determine relative levels of cellular metabolites. Relative levels of (a) amino acids and (b, c) acyl carnitines are shown. O Ilkayeva, CB Newgard contributed to this data.

4.2.4 PDHK is required for Th2 and Th17, but not Treg, function *in vitro*

The finding of distinct substrate utilization patterns and differential expression of PDH isoforms suggested that this bifurcation point for pyruvate may be an important regulatory node for CD4 subsets. PDH is a highly regulated multi-subunit complex that is difficult to target pharmacologically. However, PDH activity is controlled, in part, by PDHK, which phosphorylates and inhibits PDH to direct pyruvate to lactate rather than to acetyl CoA. Therefore, the expression of the four PDHK isoforms was examined in the CD T cell subsets. T cells expressed PDHK1 and PDHK3, with PDHK1 being the prominent isoform (**Fig 4.10a**). At both the RNA and protein level, Th2 and Th17 expressed the highest levels of PDHK1, followed by Treg, while Th1 had little PDHK1 expression (**Fig 4.10b**). Therefore, PDHK1 is differentially expressed in the CD4 T cell subsets, potentially allowing for highly selective metabolic targeting of CD4 T cell populations.

PDHK is a target of the inhibitor compound dichloroacetate (DCA), which has been previously shown to differentially affect Th17 and Treg. The inhibition of PDHK, which in turn activates PDH, turns on the critical step to convert pyruvate to acetyl CoA and drive glucose into the TCA cycle to be oxidized (**Fig 4.10c**). To determine the effect of PDHK inhibition on CD4 T cell fate and function, CD4 T cells were differentiated *in vitro* in the presence of DCA.

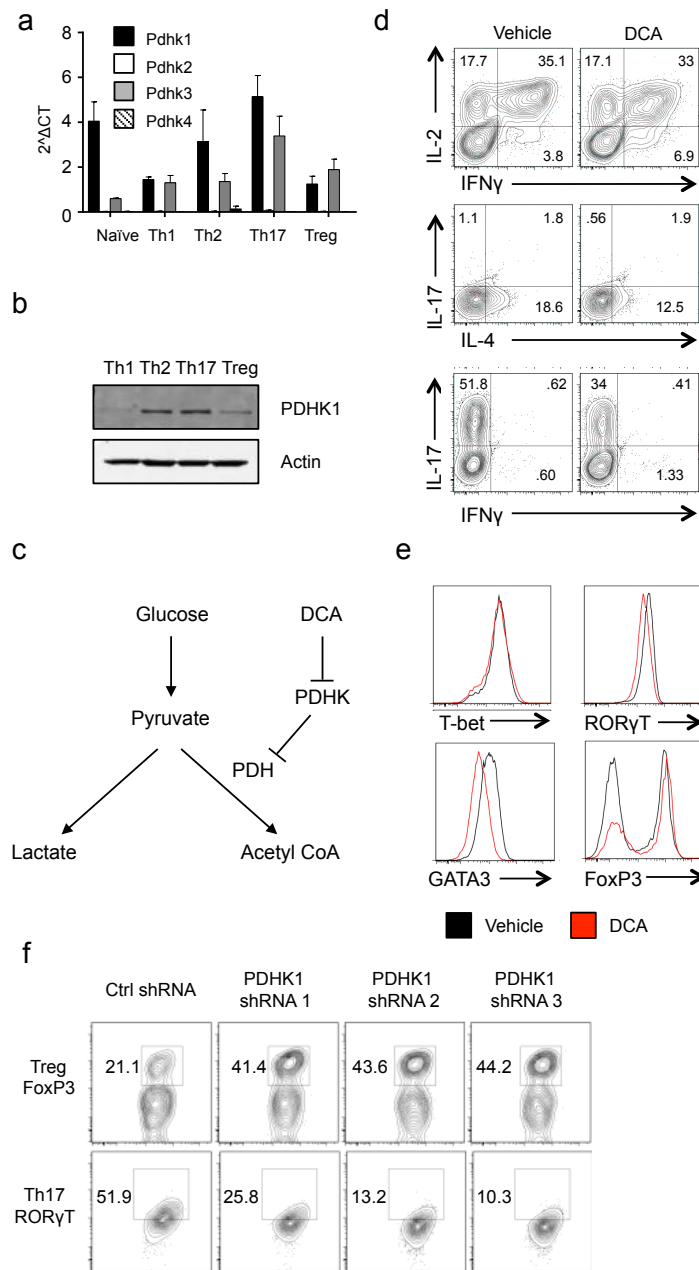


Figure 4.10: PDHK is required for Th2 and Th17, but not Treg, function *in vitro*

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells for (a) real-time PCR or (b) immunoblot. (c) A schematic showing the different fates of pyruvate and mechanism of DCA inhibition. (d, e) Cells were treated with 10mM dichloroacetate (DCA) and cytokine production (d) and transcription factor

staining (e) was determined by flow cytometry. (f) T cells were polarized, infected with lentivirus expressing PDHK1 shRNA and transcription factor staining for FoxP3 and ROR γ t was performed. Data is shown as mean \pm SD (a) and all data are representative of at least 3 independent experiments. PS Winter contributed to this data.

DCA treatment did not affect Th1 differentiation or function, as IFN γ production and T-bet expression were similar regardless of treatment (**Fig 4.10d, e**). This is likely due to the low or absent expression of PDHK in Th1. In contrast, DCA inhibited the production of IL-4 and IL-17 and suppressed expression of the Th2 and Th17 transcription factors GATA3 and ROR γ t. Surprisingly, treatment of Treg with DCA increased the expression of FoxP3 compared to the vehicle condition. This suggests that directing glucose to enter mitochondrial metabolism is favorable for Treg, yet has an adverse effect on Th2 and Th17. To confirm that the effects seen with pharmacologic inhibition of PDHK1 were not off-target drug effects, we genetically targeted PDHK1 using a lentiviral construct. Knockdown of PDHK1 using 3 different lentiviral constructs inhibited ROR γ t expression in the Th17s and increased FoxP3 expression, mimicking the *in vitro* effects seen with DCA (**Fig 4.10**). Therefore, PDHK1 plays a key role to promote Th2 and Th17 cells while suppressing Treg generation.

One outcome of DCA treatment to promote pyruvate oxidation is a potential increase in the generation of reactive oxygen species (ROS). Indeed, DCA can suppress aerobic glycolysis of cancer cells and stimulate ROS production that can lead to cancer cell senescence (Michelakis *et al.*, 2010). Previous literature also suggests that Teff may

be more sensitive to ROS stress than Treg (Mougiakakos *et al.*, 2009; Won *et al.*, 2013). ROS levels were therefore examined in the T cell subsets. The ROS indicator dye, DCFDA, showed that Treg had higher levels of ROS than Teff (**Fig. 4.11a**). Consistent with ROS stress, Treg also had a large reserve pool of reduced glutathione (GSH), as well as high levels of oxidized glutathione (GSSG) (**Fig. 4.11b**). This suggests that Treg have a greater capacity to handle ROS and utilize this GSH pool to a greater extent than Teff. Treg have also been shown to have higher levels of the antioxidant thioredoxin-1, also contributing to Treg redox regulation (Mougiakakos *et al.*, 2011).

To determine if DCA acted in part through ROS generation, established Th17 and Treg cultures were treated acutely with DCA. In both cases, DCA increased DCFDA staining to indicate generation of ROS (**Fig. 4.11c**). We next tested the effects of DCA-induced ROS by co-treatment of Th17 and Treg with DCA and the antioxidant N-acetyl cysteine (NAC) to prevent ROS accumulation. DCA treatment alone inhibited IL-17 production, however co-treatment with NAC prevented the decrease in IL-17 (**Fig. 4.11d**). In contrast, while DCA promoted Treg, co-treatment with NAC prevented the increase. These data suggest that DCA drives glucose oxidation, generating ROS that negatively impacts Th17 and promotes Treg, which have greater capacity to manage

ROS.

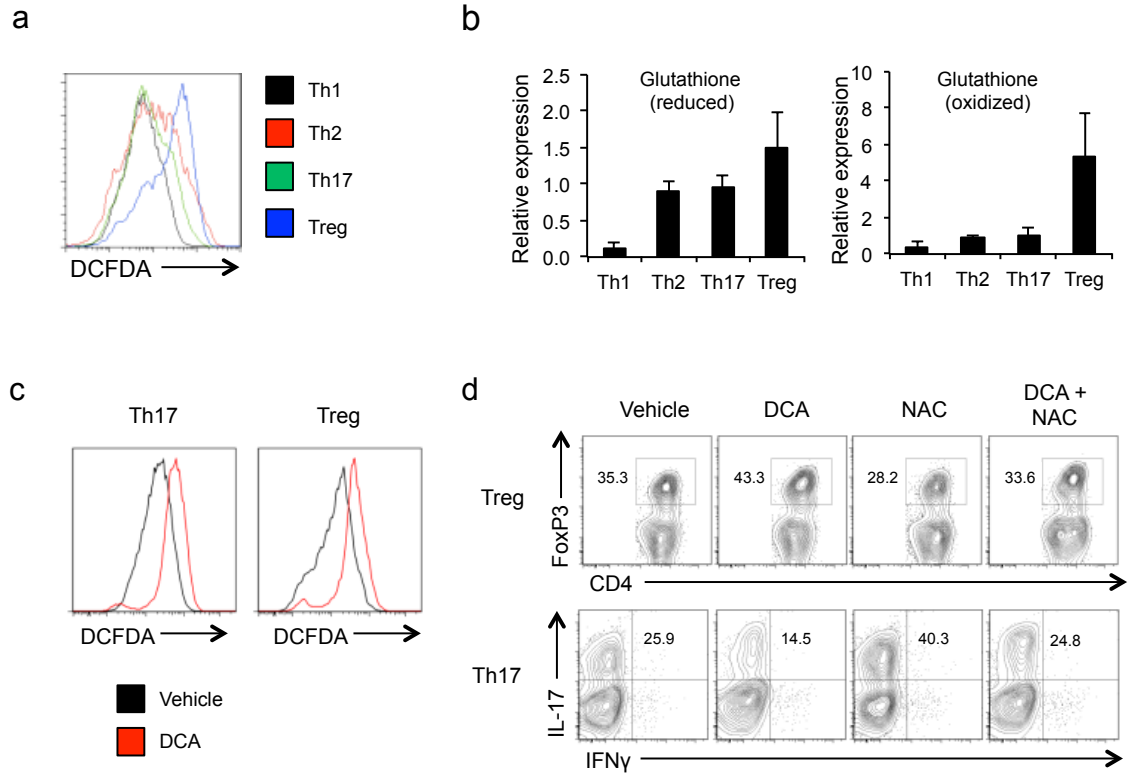


Figure 4.11: DCA treatment generates ROS that negatively impacts Th17 without affecting Treg

CD4⁺CD25⁻ T cells were polarized *in vitro* for 5 days to generate Th1, Th2, Th17 or Treg cells. (a) ROS production was measured using the indicator dye DCFDA. (b) Glutathione levels were measured in the T cell subsets using mass spectrometry. (c) ROS production in vehicle and DCA treated Th17 and Treg cells was measured using DCFDA. (d, e) Treg and Th17 cells were treated with 10mM DCA, 1mM N-acetyl cysteine (NAC) or both in combination and (d) FoxP3 and IL-17 production were examined after 3 days. Data is shown as mean \pm SD (b, e) and all data are representative of at least 2 independent experiments.

4.2.5 Inhibition of PDHK *in vivo* differentially affects Th17 and Treg to inhibit the progression of colitis and EAE

In vitro, PDHK inhibition by DCA treatment has a deleterious and selective effect on Th17 function while favoring Treg differentiation. While DCA has been previously shown to suppress inflammation in arthritis, asthma, and alloreactivity (Bian *et al.*, 2009; Ostroukhova *et al.*, 2011; Eleftheriadis *et al.*, 2013), the selective effect on Th17 and Treg, but not Th1, subsets has not been examined. We, therefore, tested the ability of DCA to suppress the Th17-mediated inflammatory diseases. IBD is driven by both Th1 and Th17 and alleviated by Treg to allow examination of each subset *in vivo* (Hale *et al.*, 2005). Naïve T cells were adoptively transferred into immunodeficient recipients in the absence of Treg and were then given normal or DCA-containing drinking water. IBD is initiated upon generation of Teff by encounter with gut microbiota. Because mice were negative for enteropathic bacterial species, the animals were given the clinically-relevant NSAID piroxicam two weeks after the T cell transfer to initiate gut damage and IBD. Significantly fewer CD4⁺ cells were found in the mesenteric lymph nodes and infiltrating into the gut tissue of mice treated with DCA relative to those receiving vehicle water (**Fig 4.12a**). Importantly, DCA treatment did not lead to a reduction of Th1 cells, as measured by IFN γ production (**Fig 4.12b**). However, IL-17 production was significantly reduced with DCA treatment (**Fig 4.12c**). These data suggest that PDHK inhibition selectively affects Th17 but not Th1 proliferation and function *in vivo*.

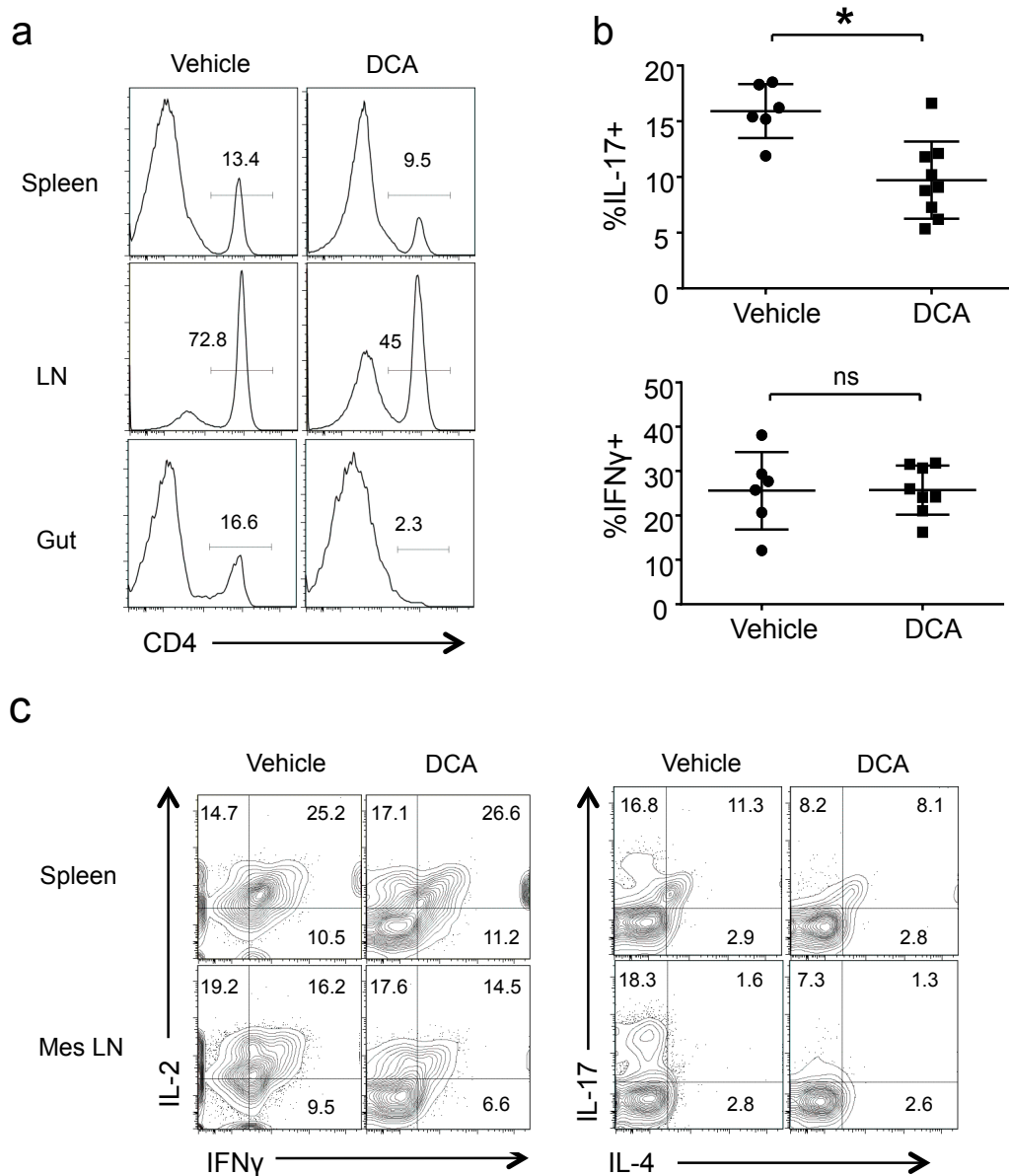


Figure 4.12: PDHK is selectively required for Th17 but not Th1 expansion and function *in vivo*

(a-c) Rag1^{-/-} mice were injected with naïve effector (CD4⁺CD25⁻CD45RB^{hi}) T cells and colitis was induced. Mice were given 2g/L of dichloroacetate (DCA) or vehicle in the drinking water for the duration of the experiment. (a) The percentage of CD4 T cells or (b, c) IL-17 or IFN γ producing cells in the spleen and mesenteric lymph nodes was determined using flow cytometry. RJ Kishton contributed to this data.

DCA treatment did not prevent intestinal inflammation or disease progression, likely due to the functional Th1 response (data not shown).

To independently test the role of PDHK on Th17 and Treg populations, we next examined the effects of DCA in EAE. While Th1 cells can contribute to EAE, this model is Th17-dependent, as ROR γ t knockout mice are disease-resistant. EAE was induced in wild type mice by MOG injection with pertussis toxin administered on days 0 and 2. DCA was again given in the drinking water throughout the course of the experiment. Clinical signs of EAE were assessed at regular intervals. PDHK inhibition by DCA treatment significantly alleviated EAE symptoms throughout the course of disease progression (**Fig 4.13a**). Importantly, DCA treatment inhibited CD44^{hi} cells while increasing FoxP3⁺ Treg (**Fig 4.13b**). Therefore, PDHK inhibition *in vivo* selectively modulates the balance between Th17 and Treg and suppresses autoimmunity in two mouse models. These data suggest that the distinct metabolic programs of CD4 subsets are both essential for each subset and can be exploited to target specific T cell populations in inflammatory diseases such as through DCA-mediated selective inhibition of Th17 cells.

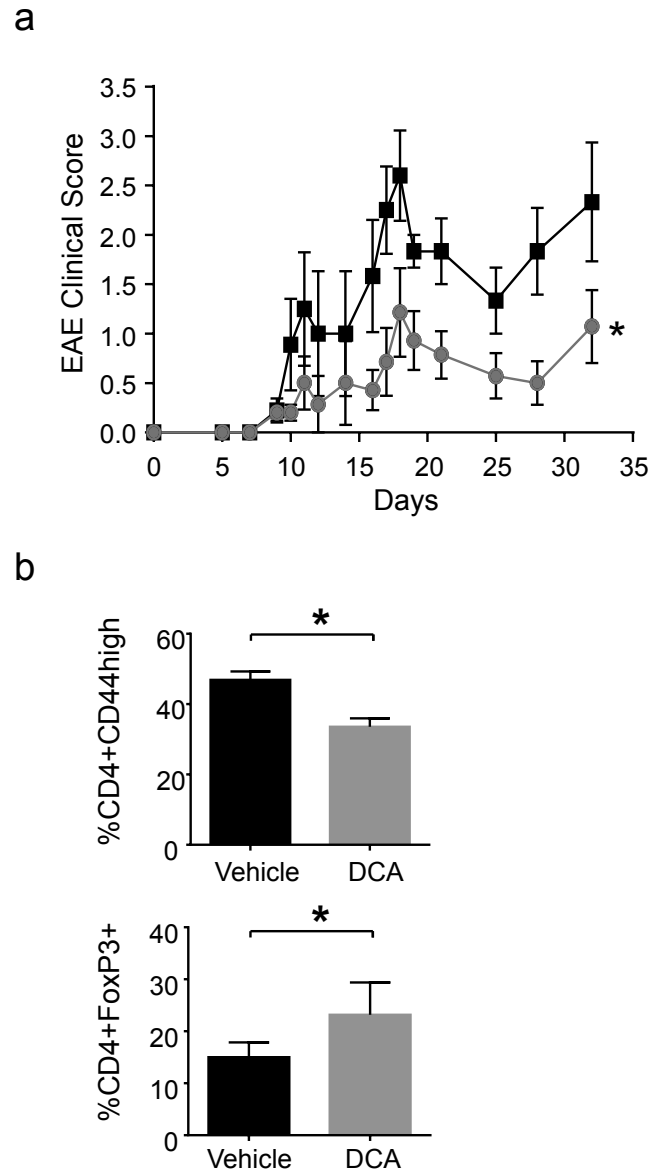


Figure 4.13: PDHK is selectively required for Teff but not Treg expansion and function *in vivo*

(a, b) EAE was induced in wildtype mice with or without DCA treatment and (a) time course of clinical score and (b) percentages of CD4⁺CD44^{hi} and CD4⁺FoxP3⁺ cells were determined using flow cytometry. Data is shown as mean \pm SD (e) and all data are representative of 2 independent experiments. AN Macintyre and M Inoue contributed to this data.

4.3 Discussion

It is becoming increasingly evident that metabolic reprogramming plays a crucial role in T cell activation, differentiation and function. Activated CD4 T cells require high rates of glucose uptake and glycolysis, glutaminolysis and lipid synthesis to support proliferation and function (Jacobs *et al.*, 2008; Kidani *et al.*, 2013; Sinclair *et al.*, 2013; Wang *et al.*, 2011). We show for the first time a detailed analysis of the metabolic programs underlying each CD4 T cell subset (Th1, Th2, Th17 and Treg). These data show that Teff are reliant on glucose while Treg have fuel flexibility and oxidize glucose in addition to lipids. However, while Th1, Th2 and Th17 have a similar need for glucose, each T cell subset has a distinct metabolic profile that may allow for selective targeting of different T cell populations. Here, we identify pyruvate dehydrogenase kinase (PDHK) as a selective regulator of CD4 T cell differentiation and inflammation.

Previous studies have identified broad metabolic differences between Teff and Treg, however the detailed metabolic profile of each cell type has not been previously examined. We show here that Treg are less efficient at the conversion of glucose to lactate compared to effector T cells. However, Treg have a higher spare respiratory capacity and preferentially oxidize fuels in the mitochondria. Importantly, this increased oxygen consumption was not due to lipid oxidation, as lipids were not present in the media. This suggested for the first time that Treg not only oxidize lipids, but also glucose. This fuel flexibility may be important *in vivo* during T cell suppression and

there is increasing evidence that Treg utilize metabolic strategies to suppress Teff (33). This includes degrading ATP through upregulating the expression of CD39, as well as directly competing with Teff for cysteine uptake (34). Treg can also induce dendritic cells to consume essential amino acids, which inhibits T cell proliferation (35). Taken together, these data argue that it would be advantageous for Treg to have a distinct metabolic profile from Teff. If Teff and Treg had similar signaling pathways and metabolism, it may be difficult for Treg to functionally inhibit Teff when faced with the same environmental cues. Therefore, the metabolic differences between Teff and Treg may be necessary for the ability of Treg to function and suppress inflammation.

In vitro 2-deoxyglucose (2DG) treatment to inhibit glycolysis prevented Teff pro-inflammatory cytokine production as well as Teff, but not Treg, survival and proliferation. Previous literature has described a role for glycolytic intermediates in the translation of IFN γ , as GAPDH can bind to IFN γ mRNA and inhibit translation (Chang *et al.*, 2013). This suggests that metabolic enzymes directly regulate inflammatory cytokine production. However, 2DG treatment also inhibited the expression of the effector transcription factors T-bet and ROR γ t. This suggests that glycolytic inhibition suppressed T cell differentiation as well as cytokine production. Therefore, T cell metabolism is tightly linked to cell fate as well as function. Treg, on the other hand, were unaffected by glycolytic inhibition. This is likely due to the ability of Treg to oxidize alternative fuels, such as lipids. In contrast, low doses of rotenone to inhibit

mitochondrial respiration only modestly affected Teff and yet strongly inhibited the survival and proliferation of Treg. Rotenone inhibits complex I of electron transport and prevents the transfer of electrons from NADH to the electron transport chain, inhibiting mitochondrial ATP generation. As Treg cannot upregulate aerobic glycolysis after mitochondrial inhibition, rotenone treatment likely inhibited Treg energy production and prevented cell growth and survival. Teff were less affected by rotenone, suggesting that they are able to generate sufficient ATP through the conversion of pyruvate to lactate in aerobic glycolysis. Rotenone has also been shown to increase reactive oxygen species (ROS) due to the inhibition of Complex I, however Treg are able to withstand high levels of ROS (Mougiakakos *et al.*, 2009; Won *et al.*, 2013) and while we cannot exclude the possibility that this may be negatively affecting Treg, our data suggest that it is likely an issue of energy production and not ROS stress.

Given that Treg utilize mitochondrial oxidation while Teff convert glucose to lactate, we then identified the branch point between glycolysis and glucose oxidation as a potential target to modulate the balance between Teff and Treg. One of the primary regulators of this branch point is pyruvate dehydrogenase kinase (PDHK). PDHK inhibits pyruvate dehydrogenase (PDH) to suppress glucose oxidation and instead promote lactate production and glycolysis. Interestingly, PDHK1 was differentially expressed in the effector T cell subsets, expressed in Th2 and Th17 but little expression in Th1. This provided an opportunity to selectively target specific CD4 T cell subtypes.

The compound dichloroacetate (DCA) inhibits PDHK and is currently under investigation in several different types of cancer (Sutendra *et al.*, 2013). DCA is thought to be effective in cancer cells because it inhibits aerobic glycolysis and instead directs glucose to be oxidized in the mitochondria. There is also evidence that DCA promotes reactive oxygen species (ROS) in several cancer models (Gong *et al.*, 2013; Stockwin *et al.*, 2010). DCA has also been studied in an immune context. Treatment of human PBMCs with DCA inhibited pro-inflammatory cytokine production and promoted FoxP3 expression in the setting of asthma and alloreactivity (Ostroukhova *et al.*, 2012; Eleftheriadis *et al.*, 2013). Additional literature has shown a role for DCA in the treatment of airway inflammation and collagen-induced arthritis in mice (Bian *et al.*, 2009; Ostroukhova *et al.*, 2012). However, these studies do not directly examine the role of PDHK or DCA in T cells and the mechanism of inflammatory cytokine production inhibition is unclear.

Our data shows that DCA inhibits Teff differentiation and cytokine production and does so by inhibition of PDHK1 and subsequent alteration of glucose utilization. Promoting glucose oxidation through PDHK1 inhibition suppresses Th17 generation, in part through the generation of ROS. Several recent reports suggest that Teff are more sensitive to ROS stress than Treg. Deletion of the antioxidant molecule peroxiredoxin II in T cells increased Treg and prevented dextran sulfate sodium induced colitis (Won *et al.*, 2013). Here, Treg were found to have high levels of the antioxidant glutathione (**Fig**

4.11b). While Treg also have high levels of ROS compared to Teff, Treg likely produce ROS during mitochondrial oxidation and are equipped with antioxidant molecules to handle this ROS generation. Our data suggest that the Th17 are less able to handle ROS stress generated by the activation of PDH and subsequent glucose oxidation, as DCA inhibited IL-17 production. However, neutralization of ROS with the antioxidant N-acetyl cysteine normalized IL-17 production. Therefore, PDHK selectively modulates the balance between Th17 and Treg, in part through ROS generation.

Previous studies have described potential therapeutic strategies to modulate the balance between Teff and Treg. Rapamycin is used clinically to treat inflammatory disease and has been shown to suppress CD4⁺CD25⁻ T cells while preserving Treg (Shin *et al.*, 2011). However, it is not always desired to suppress all of the CD4 effector T cell subsets. Each subset plays a different role in immunity and it may be therapeutically useful to inhibit one of the subtypes without affecting the others. The finding that Th1 have little expression of PDHK1 presents a unique opportunity to fine tune CD4 T cell differentiation and the immune response. Inhibition of PDHK1 did not affect T-bet expression or IFN γ production in Th1 cells but suppressed ROR γ t and IL-17 production *in vitro*. In addition, treatment of mice with DCA in the context of inflammatory bowel disease suppressed IL-17 but preserved IFN γ production. Our data therefore suggests that targeting PDHK1 inhibits Th17 differentiation and cytokine production while leaving Th1 unaffected. This may be important in the context of Th17 driven

autoimmune disorders, where it would be favorable to suppress the Th17 lineage without affecting Th1 immunity. PDHK1 inhibition also suppressed Th2 differentiation and function, and our data would suggest that DCA therefore may also be beneficial in an allergic asthma setting. Overall, this work identified key metabolic differences between the CD4 T cell subsets and established PDHK1 as a selective regulator of the Th17:Treg balance to drive inflammatory disease.

5. FoxP3 directs an oxidative metabolic program that contributes to Treg function

5.1 Introduction

CD4 T cells are an essential component of the adaptive immune system. The ability of T cells to rapidly proliferate upon TCR engagement is critical to provide protection against foreign pathogens. T cell activation and subsequent expansion creates a significant metabolic demand, as the activated T cell needs sufficient ATP energy and biosynthetic precursors to clonally expand and initiate the immune response (Fox *et al.*, 2005; Vander Heiden *et al.*, 2009). It has recently become clear that T cell metabolism can have an enormous impact on both T cell activation and differentiation (Michalek *et al.*, 2011; Pearce *et al.*, 2009; Shi *et al.*, 2011; Wang *et al.*, 2011; Zheng *et al.*, 2009). In particular, CD4 effector and regulatory T cells have distinct metabolic requirements, likely to support cellular functions specific to each cell type.

Effector T cells (Teff), including Th1, Th2 and Th17 drive the immune response and are increased in an inflammatory setting (Zhu *et al.*, 2010). In contrast, regulatory T cells (Treg) function to suppress inflammation and are important to control autoimmunity (Sakaguchi *et al.*, 2008). Along with these distinct functional roles, Teff and Treg utilize different metabolic programs (Michalek *et al.*, 2011). Like activated T cells, Teff rely on glucose and have high rates of glucose uptake and glycolysis. This is in part due to the upregulation of the glucose transporter Glut1. In contrast, Treg

express low levels of Glut1 and a low rate of glycolysis, instead utilizing lipid or glucose oxidation for energy. Importantly, targeting glucose metabolism has been shown to be effective to inhibit Teff number or function, while maintaining Treg (Michalek *et al.*, 2011; Shi *et al.*, 2011).

Inhibition of glycolysis with the hexokinase inhibitor 2-deoxyglucose (2DG) has been shown to prevent Th17 differentiation in vitro (Shi *et al.*, 2011). Additionally, treatment of mice with 2DG suppressed experimental autoimmune encephalomyelitis (EAE). 2DG treatment also promotes Treg, supporting the idea that Treg do not rely on glucose. The pyruvate dehydrogenase kinase (PDHK) inhibitor dichloroacetate (DCA) can also promote Treg and inhibit Teff proliferation and cytokine production.

Treatment of mice with DCA inhibited airway inflammation in an asthma model as well as collagen-induced arthritis in vivo (Bian *et al.*, 2009; Ostroukhova *et al.*, 2011). DCA inhibits PDHK thereby driving glucose oxidation in the mitochondria. These and other studies suggest that Teff rely on glucose while Treg have fuel flexibility but importantly require mitochondrial oxidation to generate ATP energy.

One important question that arises from these studies is what drives the Treg oxidative program. In mice, the Treg lineage is defined by the expression of the transcription factor FoxP3. FoxP3 is known to regulate a variety of genes including CTLA-4 and CD25, as well as inhibit the expression of IL-2, IFN γ and other effector molecules (Sakaguchi *et al.*, 2008). However, the role of FoxP3 is more complex in

human T cells. FoxP3 is transiently expressed by all human T cells upon TCR activation (Morgan *et al.*, 2005). This transient expression of FoxP3 in non-Treg does not appear to inhibit cytokine production, nor does it confer suppressive capacity (Tran *et al.*, 2007). Recent literature described a role for FoxP3 as a negative regulator of T cell proliferation in non-Treg human T cells. FoxP3-deficient non-Treg CD4 T cells proliferate more and produce more cytokines than wildtype cells (McMurchy *et al.*, 2012). As proliferation and metabolic status are closely linked, this suggests that FoxP3 may be restraining T cell metabolism to inhibit proliferation.

Given that Teff and Treg have distinct metabolic profiles, we hypothesized that FoxP3 may drive the Treg oxidative program. Here, we examine the role of FoxP3 in metabolism and determine that FoxP3 promotes glucose and lipid oxidation and suppresses glycolytic metabolism. Additionally, FoxP3 restrains glycolytic metabolism in human T cells upon activation. Importantly, we show promoting glycolysis by transgenic expression of Glut1 inhibits the ability of Treg to suppress. Therefore, the FoxP3-driven oxidative metabolic program is critical to Treg function.

5.2 Results

5.2.1 FoxP3 expression in human T cells restrains glycolytic phenotype

Previous studies have demonstrated that FoxP3 is transiently expressed in all human T cells upon activation (Wang *et al.*, 2007). Deletion of FoxP3 leads to enhanced

T cell proliferation and cytokine production after TCR engagement (McMurchy *et al.*, 2012). These studies suggest that FoxP3 is a negative regulator of T cell proliferation. Because the metabolic status of a T cell can modulate proliferation, the metabolic effect of FoxP3 knockdown was examined in human T cells. Knockdown of FoxP3 increased glucose uptake and glycolysis in activated T cells (**Fig. 5.1A, B**). Metabolic protein expression was also examined after FoxP3 knockdown. In the majority of donors, the expression of the glucose transporter Glut1, as well as hexokinase 2 (Hk2) and the mTor target phospho-S6 were upregulated upon FoxP3 deletion (**Fig 5.1C, donors 1 and 2**). This suggests that FoxP3 inhibits glycolytic metabolism in human T cell activation and this may contribute to its role as a negative regulator of proliferation.

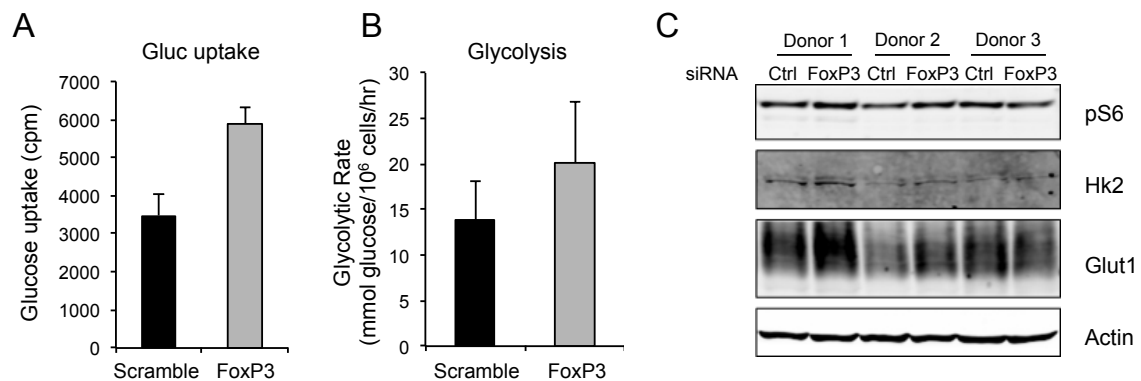


Figure 5.1: FoxP3 restrains glycolysis in human T cell activation

Peripheral blood T cells from healthy donors were transfected with scramble siRNA or FoxP3 siRNA and stimulated with anti-CD3 and anti-CD28 for 48 hours. Data show (A) glucose uptake and (B) glycolytic rate after 48 hours. (C) Immunoblotting on samples collected after scramble or FoxP3 siRNA from 3 different healthy donors. Data is representative of at least 6 replicates. (A) and (B) show the mean \pm SD.

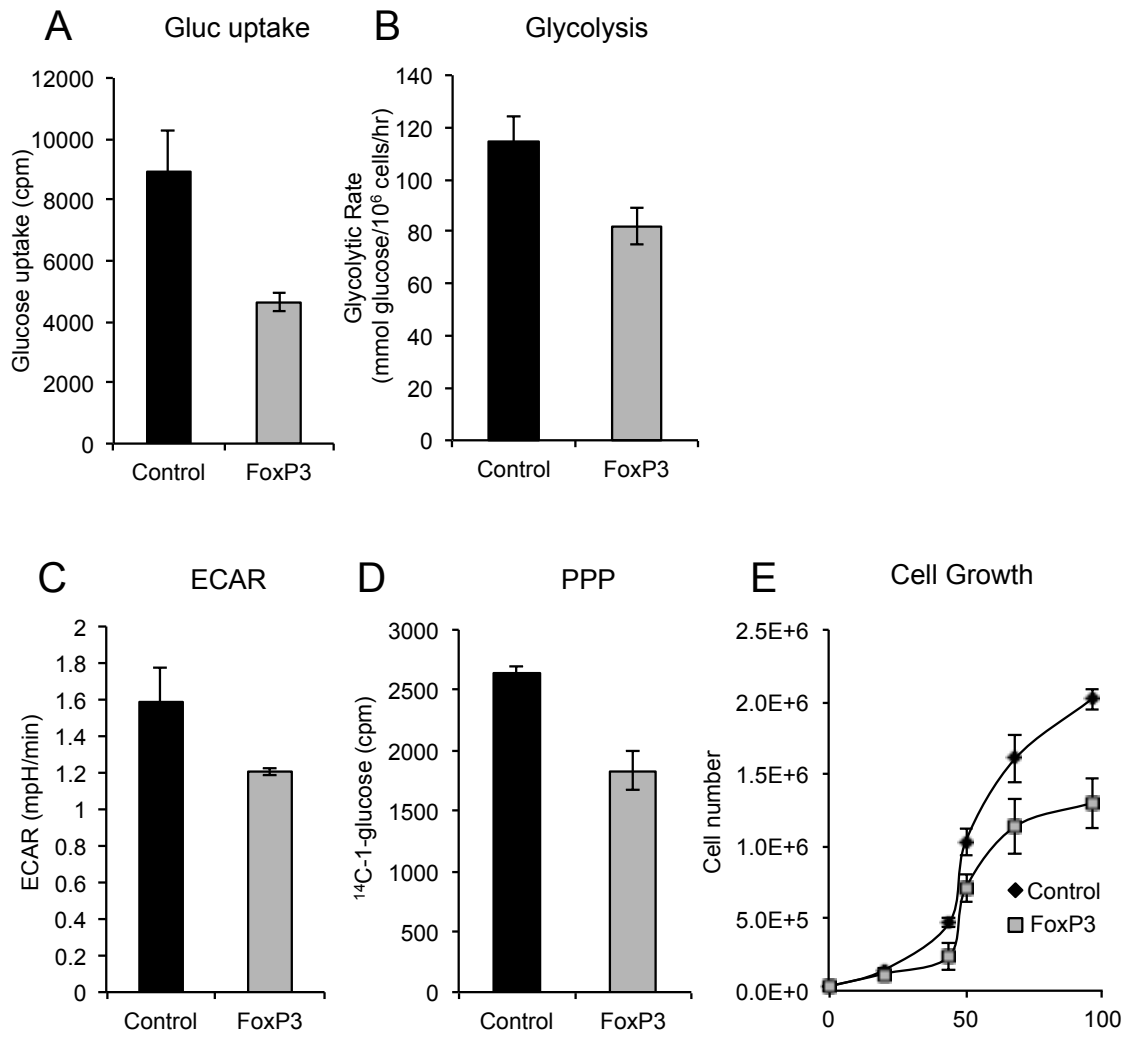


Figure 5.2: FoxP3 expression suppresses glycolytic metabolism and growth

FL5.12 pro-lymphocyte cells were lentivirally infected with FoxP3-ER-NGFR or control NGFR vector. 3 FoxP3-ER and 3 control lines were treated with 4-OHT for 36 hours and then examined for (A) glucose uptake, (B) glycolysis, (C) extracellular acidification rate (ECAR), (D) pentose phosphate pathway (PPP) and (E) cell growth over the course of 4 days. Data is representative of 3 independent experiments. (A-E) show the mean \pm SD. AG Nichols and RJ Kishton contributed to this data.

5.2.2 Expression of FoxP3 drives an oxidative metabolic program

Our data suggests that FoxP3 inhibit glycolytic metabolism in human T cells. This is significant given the emerging data about differences between Teff and Treg metabolism. While Teff utilize a glycolytic program, Treg are not reliant on glucose and we have previously showed that Treg have high levels of fatty acid oxidation (Michalek *et al.*, 2011). We therefore examined the role of FoxP3 in metabolism using an inducible FoxP3 construct. In this model, FoxP3 is conjugated to a fragment of the estrogen receptor and remains in the cytoplasm until 4-hydroxytamoxifen (4-OHT) is added to translocate the FoxP3-ER to the nucleus where FoxP3 can initiate its transcriptional activity (Allan *et al.*, 2008). The FoxP3-ER cell lines were generated using the FL5.12 prolymphocyte cell line, which has been used previously to model T cell metabolism (Wofford *et al.*, 2008; Rathmell *et al.*, 2000). After 36 hours of 4-OHT treatment, the metabolic profile of three FoxP3-ER clones and three control clones were examined. The cells expressing FoxP3 had suppressed glycolytic measurements, including glucose uptake, glycolysis and extracellular acidification rate (ECAR) which is a measurement of lactate production (**Fig. 5.2A-C**). FoxP3-expressing cells also had decreased flux through the pentose phosphate pathway (PPP), which contributes to nucleotide and lipid synthesis (**Fig. 5.2D**). These cells also had a striking growth defect, likely due to the inhibition of glycolytic metabolism and PPP (**Fig. 5.2E**). Taken together, these data suggest that FoxP3 expression inhibits glycolytic metabolism and slows cell growth.

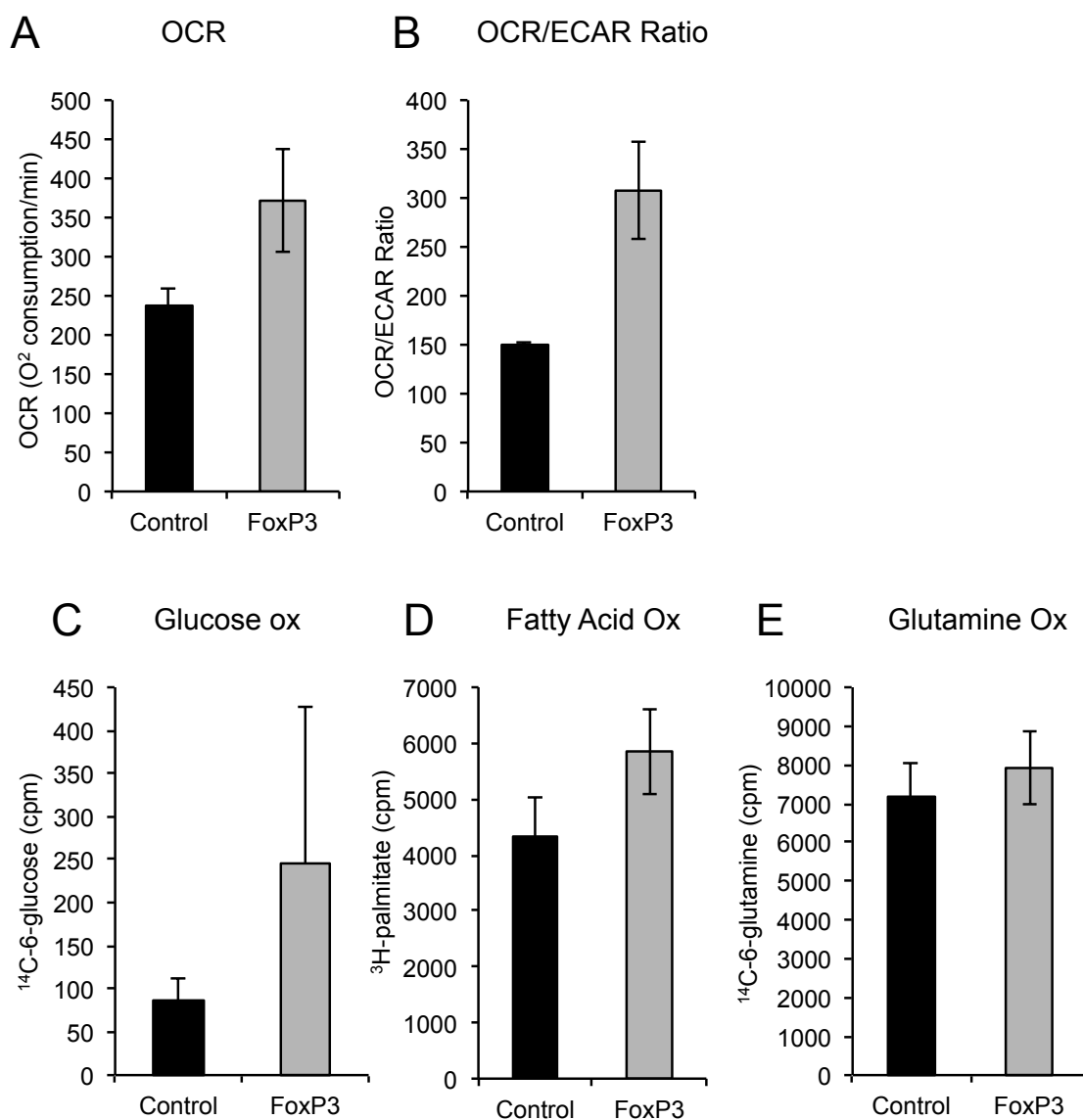


Figure 5.3: FoxP3 expression promotes glucose and lipid oxidation

FL5.12 pro-lymphocyte cells were lentivirally infected with FoxP3-ER-NGFR or control NGFR vector. 3 FoxP3-ER and 3 control lines were treated with 4-OHT for 36 hours and then examined for (A) oxygen consumption rate, (B) the ratio of OCR to ECAR, (C) glucose oxidation, (D) fatty acid oxidation and (E) glutamine oxidation. Data is representative of 3 independent experiments. (A-E) show the mean \pm SD. RJ Kishton contributed to this data.

These cells, however, were not metabolically inactive. While glycolytic metabolism was suppressed, oxidative metabolism was increased. FoxP3-expressing cells had higher oxygen consumption (OCR) and an increased ratio of OCR to ECAR (**Fig. 5.3A, B**). This was likely due to an increase in glucose and lipid oxidation (**Fig. 5.3C, D**). In contrast, glutamine oxidation was unchanged (**Fig 5.3E**). Therefore, expression of FoxP3 promotes an oxidative metabolic program and inhibits glycolysis and PPP, mirroring the Treg metabolic phenotype.

Because this work was done in a lymphocyte cell line, FoxP3 was also retrovirally expressed in primary CD4⁺CD25⁻ T cells to examine the effect on metabolism. Similarly to the cell line, FoxP3 expression in primary T cells inhibited glucose uptake (**Fig 5.4A**). Although glycolysis was decreased upon FoxP3 expression (**Fig. 5.4B**), this measures flux through the enolase step of glycolysis and may represent increased glucose oxidation. Indeed, OCR and the ratio of OCR to ECAR were increased in primary T cells expressing FoxP3 (**Fig. 5.4C, D**). Overall, this data suggests that the Treg-specific transcription factor FoxP3 promotes an oxidative metabolic phenotype in T cells (**Fig 5.5**).

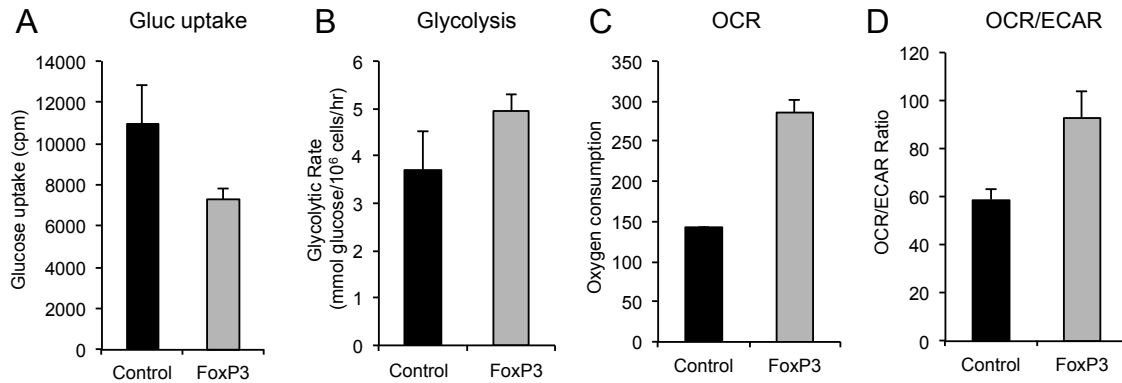


Figure 5.4: Retroviral expression of FoxP3 in primary T cells drives an oxidative metabolic program

CD4⁺CD25⁻ primary mouse T cells were retrovirally infected with FoxP3-NGFR or control NGFR. T cells were activated for 72 hours and then examined for (A) glucose uptake (Gluc uptake), (B) glycolysis, (C) oxygen consumption (OCR) and (D) the ratio of oxygen consumption to extracellular acidification rate (OCR/ECAR ratio). Data is representative of 3 independent experiments. (A-D) show the mean \pm SD. AG Nichols and RJ Kishton contributed to this data.

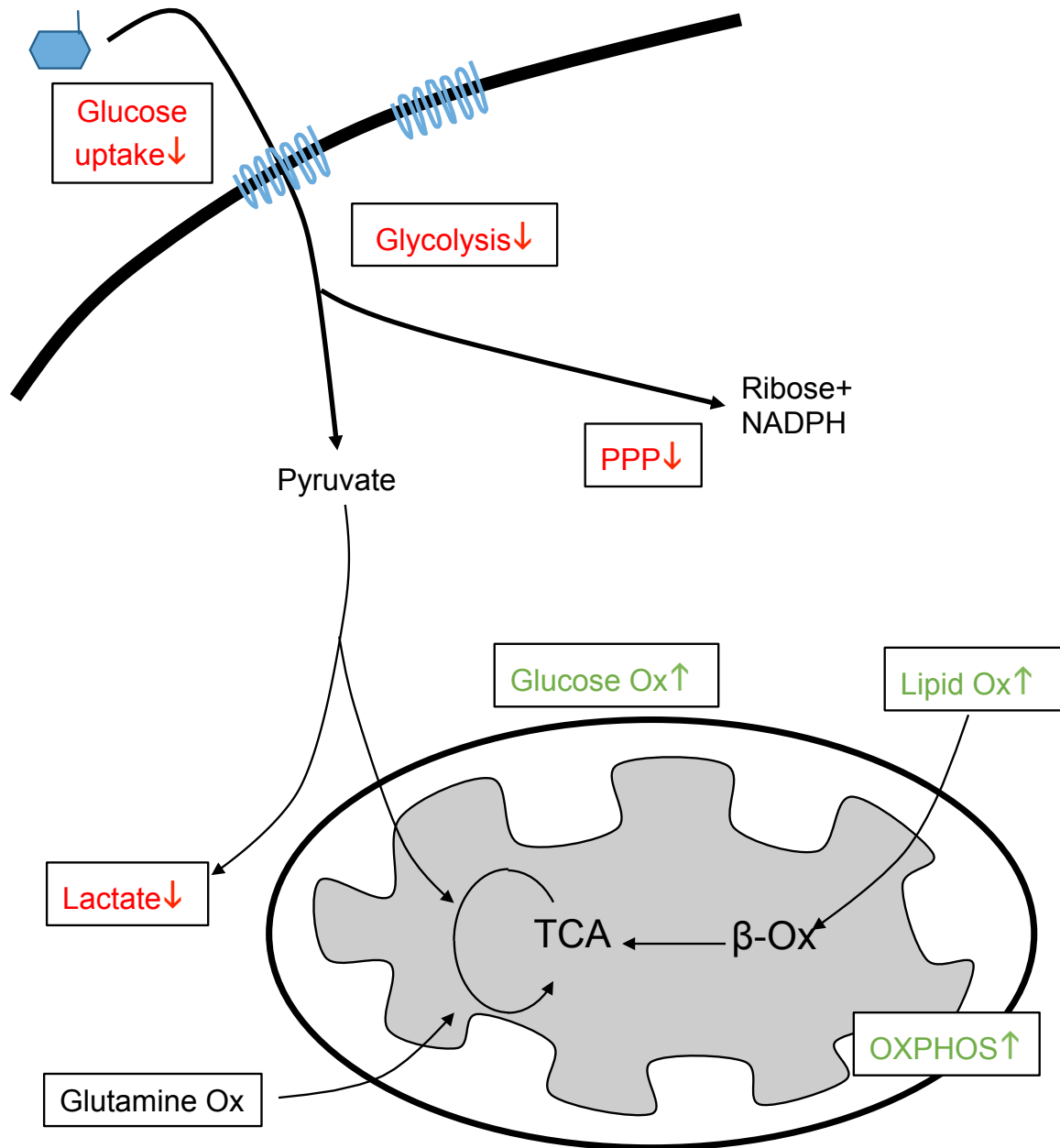


Figure 5.5: Overview of metabolic changes driven by FoxP3

FoxP3 expression leads to metabolic changes including reduced glucose uptake, glycolysis, pentose phosphate pathway (PPP) and decreased lactate as measured by extracellular acidification rate. Additionally, FoxP3 promotes glucose oxidation, lipid oxidation and oxygen consumption. Glutamine oxidation was unchanged.

5.2.3 Expression of FoxP3 alters metabolic gene expression

FoxP3 is a transcription factor that is known to interact with NFAT, NFκB and other transcriptional regulators to promote or suppress gene transcription and drive the Treg lineage (Wu *et al.*, 2006). Many studies have examined FoxP3 target genes, however these were focused on the immune aspects of FoxP3's regulatory network (Zheng *et al.*, 2007). Our data suggest that FoxP3 drives an oxidative metabolic program but this may be due to additional transcription factors or secondary effects. We therefore wanted to examine the role of FoxP3 on metabolic gene expression. FoxP3 was retrovirally expressed in primary CD4⁺CD25⁻ T cells and gene expression was examined. Pathway analysis of affymetrix data showed that lipid metabolic genes were upregulated by FoxP3 while glucose and nucleotide metabolic genes were downregulated (**Fig. 5.6**). These genes appear to be both direct and indirect FoxP3 targets. Therefore, FoxP3 regulates a metabolic network both by directly targeting metabolic gene transcription and inducing secondary pathways that are regulated by FoxP3 and other transcription factors.

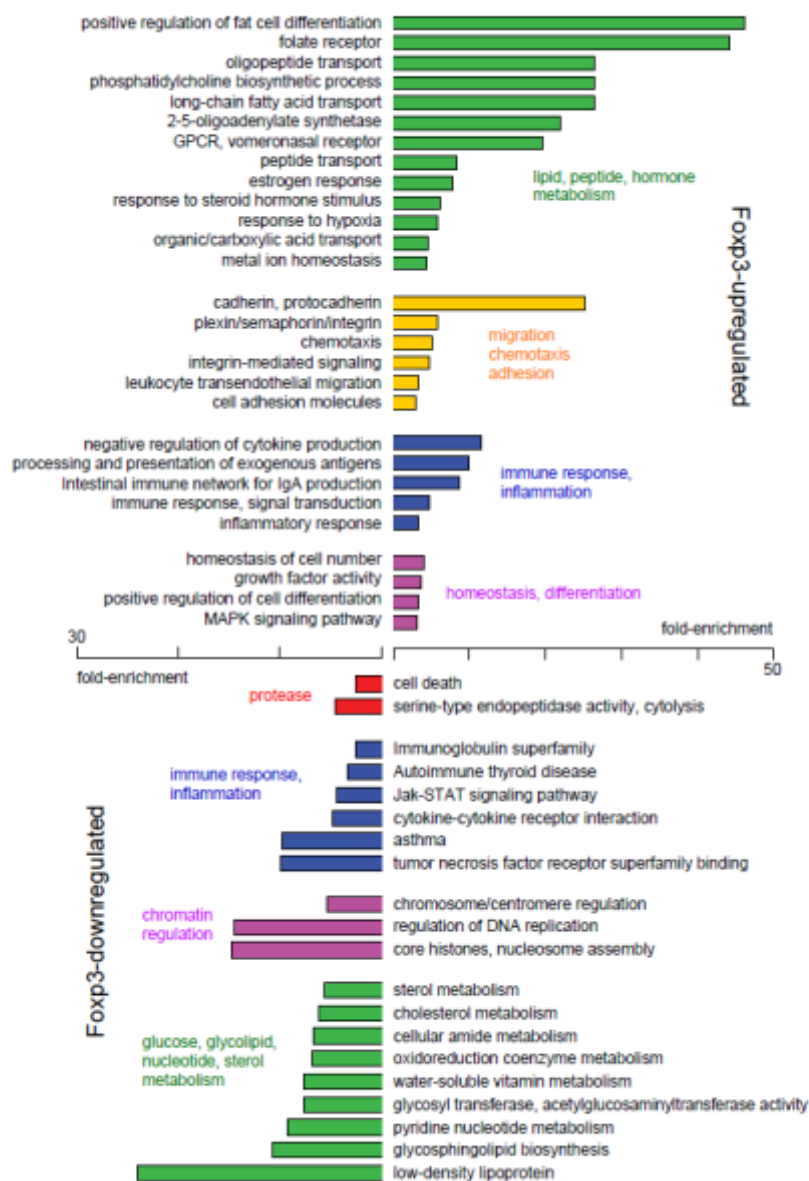


Figure 5.6: Schematic of pathways regulated by FoxP3 expression

CD4⁺CD25⁻ primary mouse T cells were retrovirally infected with FoxP3-NGFR and gene expression changes were examined. Pathways that are up or downregulated by FoxP3 expression are shown. Data contributed by AD Wells.

5.2.4 Promoting glycolysis inhibits Treg suppressive capacity

There is increasing evidence that the metabolic profiles of Teff and Treg support T cell differentiation and function. Our previous data shows that metabolic inhibition can alter T cell cytokine production, proliferation and survival (**Fig. 4.1, 4.2**). Treg utilize an oxidative metabolic program and inhibition of electron transport with rotenone limits Treg differentiation and survival. In contrast, promoting glucose oxidation by activating pyruvate dehydrogenase (PDH) activity promoted Treg differentiation and function *in vitro* and *in vivo* (**Fig. 4.11, 4.13**). This suggests that Treg function is intimately linked to an oxidative metabolic status. Therefore, we examined the effect of a glycolytic metabolism on Treg function.

We have previously described mice that transgenically express the glucose transporter Glut1 specifically in the T cells (Jacobs *et al.*, 2008; Michalek *et al.*, 2011). T cells in which Glut1 is overexpressed show increased glucose uptake, glycolysis and cytokine production. T cells from aged Glut1 transgenic mice (Glut1 tg) have elevated levels of activation markers including CD25, CD44 and CD69 and develop a mild inflammatory disorder. T cells from Glut1 transgenic mice were therefore *in vitro* differentiated into Treg and assessed for suppressive capacity. Surprisingly, Glut1 tg Treg differentiation was normal, even enhanced, compared to control Treg (**Fig. 5.7A**). However, despite increased Treg numbers, Glut1 tg Treg had decreased suppressive capacity at every ratio of effector T cells (**Fig. 5.7B**). While the functional defect is not

severe, this data suggests that promoting glycolysis in Treg is unfavorable and inhibits Treg function.

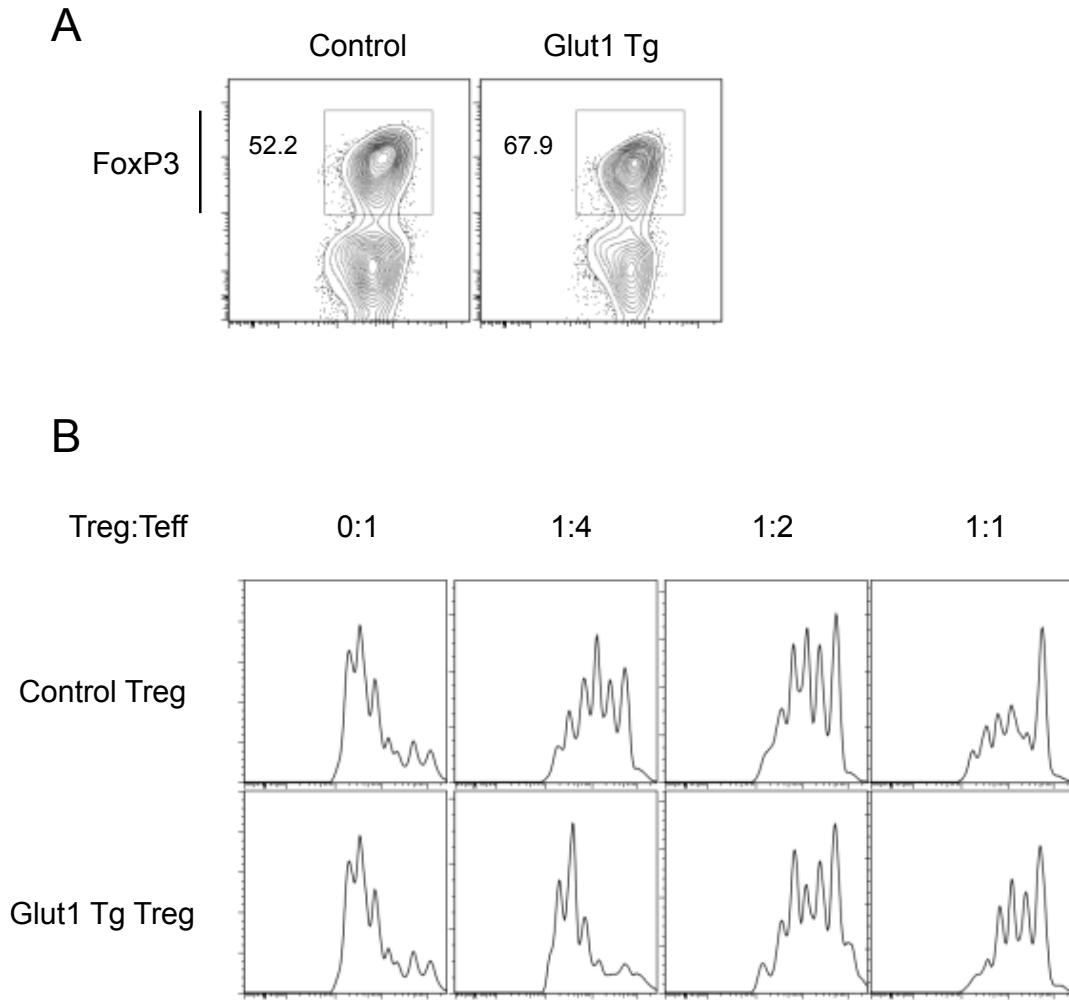


Figure 5.7: Glut1 transgenic Treg have decreased suppressive function

CD4⁺CD25⁻ T cells from Glut1 transgenic or littermate control mice were polarized *in vitro* for 5 days to generate Treg cells. (a) FoxP3 expression was examined using flow cytometry. (b) Treg function was tested in an *in vitro* suppression assay. Data is representative of 2 independent experiments.

5.3 Discussion

CD4 T cell activation leads to rapid growth, proliferation and differentiation into effector (Teff) or regulatory (Treg) cells, each with specific immune functions. Treg, in particular, are essential to maintain self-tolerance and actively suppress autoimmunity. It has recently become clear that Teff and Treg utilize distinct metabolic programs and these programs are required for the survival, proliferation and function of each subset. While Teff rely on high rates of glycolysis, Treg instead require mitochondrial oxidation and can utilize both glucose and lipids as fuel. However, the regulation of these metabolic programs is unknown. Here, we identify FoxP3 as the key regulator of Treg metabolism. Inducible expression of FoxP3 in a lymphocyte cell line or in CD4⁺CD25⁻ primary T cells suppresses glycolytic metabolism and promotes glucose and lipid oxidation. Chromatin immunoprecipitation experiments suggest that FoxP3 binds to and directly regulates the expression of many metabolic genes. Importantly, this oxidative program is required for Treg function, as overexpression of the glucose transporter Glut1 to promote glycolytic metabolism inhibits Treg function *in vitro*. Overall, this data suggests that FoxP3 drives an oxidative metabolic program that is critical to Treg function.

The transcription factor FoxP3 is considered to be the master regulator of Treg and is critical to Treg development and function (Fontenot *et al.*, 2003; Hori *et al.*, 2003). FoxP3-deficient mice lack Treg and develop a fatal multiorgan autoimmune disease

(Fontenot *et al.*, 2003). While FoxP3 appears to be the key regulator of mouse Treg, the role of FoxP3 in human T cells is less clear. FoxP3 is not sufficient to confer the human Treg phenotype and human Treg are now defined by multiple transcription factors, including FoxP3, Helios, Eos and others (Thornton *et al.*, 2010; Sharma *et al.*, 2013). Additionally, FoxP3 is transiently expressed by a majority of CD4 and CD8 conventional T cells immediately following T cell activation (Walker *et al.*, 2003). This expression of FoxP3 does not necessarily lead to a suppressive phenotype, as many FoxP3⁺ human T cells still produce IL-2 and IFN γ and cannot suppress (Allan *et al.*, 2007; Gavin *et al.*, 2006). Together, these data suggest that FoxP3 may have functions beyond Treg suppression.

A recent study showed that FoxP3 knockdown increased proliferation and cytokine production in conventional T cells (McMurchy *et al.*, 2013). Here, we show that FoxP3-null human T cells have increased glycolytic metabolism and glycolytic protein expression. As metabolism and proliferation are tightly linked (Fox *et al.*, 2005; Vander Heiden *et al.*, 2009), our data suggests that FoxP3 restricts glycolytic metabolism and thereby prevents proliferation in conventional human T cells. Interestingly, FoxP3 may be differentially localized in Treg and conventional T cells. Magg *et al.* showed that FoxP3 can shuttle between the nucleus and cytoplasm and is largely located in the cytoplasm in conventional T cells (Magg *et al.*, 2012). Whether FoxP3 is directly

regulating metabolic changes through gene expression or through another mechanism is still under active investigation.

We and others have shown that Teff and Treg utilize distinct metabolic pathways and these pathways are critical for the survival, proliferation and function of each subtype (Michalek *et al.*, 2011b; Shi *et al.*, 2011). The regulation of these metabolic programs, however, is unknown. Here, we examined the role of FoxP3 in Treg metabolism and found that FoxP3 drives an oxidative metabolic program that is critical to Treg function. While our gene expression data suggests that FoxP3 directly regulates metabolic gene expression, it is likely that FoxP3 functions to drive these metabolic changes through both direct and indirect methods and will be examined in further studies.

Several other signaling pathways have been shown to modulate the balance between Teff and Treg and may interact with FoxP3 to contribute to the Treg metabolic phenotype. Delgoffe *et al.* showed that mTOR knockout T cells fail to become Teff even when differentiated *in vitro* with the appropriate cytokines and instead default to the Treg lineage (Delgoffe *et al.*, 2009). The mTOR pathway affects a variety of signaling pathways, including metabolic pathways, and may be an important contributor towards Teff and Treg metabolic profiles (Chi, 2012). Future work is needed to determine if FoxP3 and mTor interact to contribute to the Treg metabolic phenotype. Additionally, there is increasing evidence that Treg may use metabolic strategies to suppress Teff

(Shevach, 2009). Treg can trigger a signaling pathway in dendritic cells that inhibits glutathione (GSH) synthesis to inhibit Teff (Yan *et al.*, 2010). Treg can also induce dendritic cells to consume essential amino acids (EAA), which inhibits T cell proliferation (Cobbold *et al.*, 2009). Taken together, these data argue that it would be beneficial for Treg to utilize a different metabolic program than Teff.

While previous studies showed that Treg utilize mitochondrial oxidation for energy production, this is the first to show that promoting glycolytic metabolism suppresses Treg function. Glut1 transgenic T cells have been previously shown to have increased glucose uptake as well as increased cytokine production in aged mice (Jacobs *et al.*, 2008). While *in vitro* differentiation into FoxP3⁺ Treg was normal or even enhanced in Glut1 transgenic T cells compared to vehicle, these cells were less suppressive in an *in vitro* suppression assay. Interestingly, Zeng *et al.* showed that Treg can also be generated from Raptor-deficient (mTORC1-deficient) T cells but they are not functional (Zeng *et al.*, 2013). mTORC1 deletion was also shown to suppress lipid metabolism and the rate of oxygen consumption. Overall, these data suggest that FoxP3 drives an oxidative metabolic program in Treg that is distinct from Teff metabolism. These metabolic differences between Teff and Treg may be necessary for the ability of Treg to function and suppress inflammation during an active immune response.

6. Conclusion and Future Directions

The work described here outlines four major points. 1) Inhibition of glucose metabolism through a T cell-specific genetic deletion of Glut1 suppresses effector T cell (Teff) but not regulatory T cell (Treg) differentiation and function. 2) While Teff generally rely on glucose and Treg are glucose independent, the four T cell subsets; Th1, Th2, Th17 and Treg all have distinct metabolic profiles. This allows for the exciting possibility to target a specific T cell subtype in a pathological setting. 3) Inhibition of PDHK1 selectively suppresses Th2 and Th17 function, and promotes Treg differentiation. This suggests that promoting glucose oxidation is unfavorable for Th2 and Th17, while it is advantageous for Treg. 4) The Treg-specific transcription factor FoxP3 is important to direct an oxidative metabolic program that contributes to Treg function.

6.1 Glucose metabolism is required for Teff, but not Treg

While the metabolic reprogramming that occurs upon T cell activation is well described, less is understood about the metabolic phenotype of differentiated CD4 T cells (Teff: Th1, Th2, Th17; and Treg). Furthermore, the studies that suggest metabolic differences between Teff and Treg are largely *in vitro* experiments or use pharmacological agents *in vivo*, which affect all cell types, including antigen-presenting cells, and may have indirect effects on T cell differentiation. Here, a mechanistic, cell-

intrinsic approach was taken to determine the direct effect of glucose metabolism on T cell differentiation.

To determine the role of glucose metabolism on T cell differentiation, the glucose transporter Glut1 was genetically deleted specifically in T cells. Glut1 is one of the major transporters in T cells (**Fig. 3.1B**) and is upregulated and trafficked to the cell surface during T cell activation (Jacobs *et al.*, 2008; Wofford *et al.*, 2008). Importantly, Glut1 represents one major entry point of glucose into the cell and therefore deletion of Glut1 suppresses global cellular glucose metabolism. By deleting Glut1 specifically in T cells using a T cell specific Cre (LckCre or CD4Cre), we were able to determine the cell-intrinsic role of glucose metabolism. Indeed, deletion of Glut1 in T cells led to decreased glucose uptake, glycolysis and lactate production and proliferation upon T cell activation (**Figs. 3.2 and 3.3**). Deletion of Glut1 in the T cell compartment led to a drastic increase in the percentage of T cells that were FoxP3⁺ (**Fig. 3.4A**). As the number of FoxP3⁺ Treg was unchanged, this increased percentage was due to a decrease in the non-Treg T cells (**Fig. 3.4B**). In an *in vivo* model of colitis, Glut1 knockout Teff were less able to expand or function compared to control Teff, however Glut1 knockout Treg had a similar suppressive capacity as wildtype Treg.

While this work demonstrates the role of Glut1 in Teff, but not Treg, function, many questions remain to be addressed. We chose to delete Glut1 because it is at the top of the glucose metabolic pathway and will therefore globally suppress glucose

metabolism. However, Glut1 has many functions and may be affecting other aspects of T cell biology and signaling pathways besides glucose metabolic pathways. For example, Glut1 has been shown to transport ascorbic acid (vitamin C) in addition to glucose (KC *et al.*, 2005). It is possible that the lack of this important antioxidant affects Teff but not Treg function. Additionally, Glut1 has been shown to transport glucosamine, a precursor for O-linked glycosylation of proteins (Uldry *et al.*, 2002) and this cannot be ruled out as a mechanism for the effect of Glut1 on Teff with the current study.

Therefore, it will be important to examine other glucose transporters as well as metabolic enzymes further downstream of glucose transporters. Glut3 is expressed in naïve T cells and then similarly to Glut1, it is highly upregulated in Teff (**Fig. 3.1B**). However, it has a low expression level in activated T cells and Treg. This is in contrast to Glut1, which is upregulated during T cell activation and remains high in Teff. It remains to be seen whether Glut3 protein levels decrease upon initial T cell activation and later increase, or alternatively the cytokines present during *in vitro* T cell differentiation may promote the continued expression of Glut3. Regardless, it will be important to examine Glut3 deletion as an alternative strategy to inhibit glucose uptake, as it does not transport ascorbic acid and therefore any differences between Glut3 and Glut1 deletion will indicate Glut1-specific effects. This could be done using Glut3^{fl/fl}CD4Cre mice in which Glut3 is deleted specifically in the T cell compartment.

One caveat will be the potential loss of naïve T cells, as they may rely on Glut3 for glucose import and low T cells numbers would make these studies more difficult. As an alternative strategy, Glut3^{fl/fl}UbiCreER mice could be generated and therefore Glut3 could be deleted after T cell activation and the role of Glut3 could be assessed in activated and differentiated T cells.

In addition to studying the effect of deleting other glucose transporters, it is critical to examine the role of metabolic enzymes further down the glycolytic pathway. One potentially exciting target is immediately downstream of glucose import: Hexokinase (Hk). Hexokinase phosphorylates glucose to glucose-6-phosphate and analysis of Hk inhibition either pharmacologically or genetically will be important to determine if the effects on T cell function seen with Glut1 knockout T cells is specific to glucose transporter inhibition or general glycolytic inhibition.

Additionally, there are four isoforms that have distinct tissue specificity and enzyme kinetics. In T cells, Hk1 is expressed in naïve T cells and Treg while Hk2 is expressed in activated T cells and T cell (Fig. 4.4; Wang *et al.*, 2012). While Hk1 is expressed in many tissue types, Hk2 is more selectively expressed. Hk2 has also been associated with an aerobic glycolysis phenotype and is typically expressed by highly proliferative tissues, including many types of cancer (Mathupala *et al.*, 2006). Indeed, recent literature suggests that Hk2 may be a therapeutic target for cancer, as Hk2 ablation inhibited tumor initiation and maintenance in mouse models of lung and breast

cancer (Patra *et al.*, 2013). Further, mice heterozygous for Hk2 do not have changes in glucose levels when fed a normal diet, suggesting that Hk2 inhibition may be possible therapeutically (Heikkinen *et al.*, 1999). The differential expression of Hk isoforms in T cells therefore makes it a potential target for modulating the Teff:Treg balance. The lack of Hk2 expression in naïve T cells and Treg suggests that Hk2 knockout T cells may have a similar phenotype to Glut1 knockout T cells. If there are differences seen between Glut1 and Hk2 knockout T cells, it suggests that there may be Glut1- or Hk2-specific effects on T cell signaling that could then be examined in greater detail. If Hk2 looks like a promising target, there are pharmacological strategies that can also be employed. There are several drugs that target Hk including lonidamine, which is in clinical trials (Floridi *et al.*, 1998; Pelicano *et al.*, 2006). Although Hk1 and Hk2 are structurally similar, they differ in the response to inorganic phosphate and this may lead to a specific inhibition strategy (Wilson *et al.*, 2003). Therefore, inhibition of alternate glucose transporters or other metabolic enzymes such as hexokinase will provide additional evidence for the role of glucose metabolism on T cell differentiation.

Another important area of study will be to extend this work to human T cells. Although human and mouse T cells show a similar metabolic reprogramming upon activation, Teff and Treg metabolism in human T cells is unknown. To proceed forward with therapeutic strategies for human disease, it is critical to determine whether the metabolic programs we have described in mouse T cells are similar in human T cells. A

few studies have examined the role of DCA in human T cells and it appears to have a similar effect to mouse T cells (Ostroukhova *et al.*, 2012; Eleftheriadis *et al.*, 2013).

However, there are currently no studies that look at the metabolic profile of human T cells or the effect of metabolic inhibition on Teff or Treg function. There are several difficulties with working with human T cells. Whereas mouse studies use very genetically similar animals and therefore have less biological variability, humans are much more genetically diverse and there may be vast differences between the T cells of different humans. This will likely require a high number of human donors to analyze when looking at the T cell metabolic profiles.

Additionally, and unlike mouse Treg cells that are simply defined by the transcription factor FoxP3, human Treg are described by multiple transcription factors, including FoxP3, Helios, Eos and others (Thornton *et al.*, 2010; Sharma *et al.*, 2013). In addition, some groups define natural human Treg as Helios⁺ but others argue that there are both Helios⁺ and Helios⁻ natural human Treg (Himmel *et al.*, 2013). Therefore, the role of metabolism in human T cells is complicated but it is important to extend these studies to human T cells as the next step towards an application to human autoimmune disease.

6.2 Distinct metabolic profiles and requirements in Teff and Treg

An important question that arises from this work is why Teff and Treg would require distinct metabolic programs. Our work and the work of other groups suggest that Treg can function in the absence of glucose and can utilize fatty acids to support their functions (Michalek *et al.*, 2011a; Shi *et al.*, 2011). Importantly, studies with DCA indicate that Treg can utilize glucose through glucose oxidation pathways. Overall, this suggests that Treg have metabolic fuel flexibility and are able to utilize either glucose or fatty acids. Treg function to suppress Teff proliferation as a means to restrain Teff inflammatory function and prevent excessive inflammation or autoimmunity. This fuel flexibility may be important *in vivo* during T cell suppression.

Recently, there is increasing evidence that Treg utilize metabolic strategies to suppress Teff (Shevach 2009). Treg upregulate the expression of CD39, which converts ATP into AMP, thereby degrading ATP in the microenvironment and leading to Teff suppression (Deaglio *et al.*, 2007). Treg also directly compete with Teff for cysteine uptake as well as trigger a signaling pathway in dendritic cells that inhibits GSH synthesis to inhibit Teff (Yan *et al.*, 2010). Additionally, Treg can induce dendritic cells to consume essential amino acids (EAA), which inhibits T cell proliferation (Cobbold *et al.*, 2009). In this study, Cobbold *et al.* showed that amino acid starvation also inhibits the mTOR/PI3K pathway and induces Treg in a feed-forward loop. Taken together,

these data argue that it would be advantageous for Treg to have a distinct metabolic profile from Teff. If Teff and Treg had similar signaling pathways and metabolism, it would be difficult for Treg to migrate to a hostile, inflammatory environment and functionally inhibit Teff. For Treg to suppress Teff by inhibiting GSH synthesis, it suggests that Treg do not rely as heavily on GSH synthesis. For Treg to suppress by inhibiting EAA, it suggests that Treg have a different amino acid requirement than Teff. Therefore, the metabolic differences between Teff and Treg may be necessary for the ability of Treg to function and suppress inflammation.

Given our data that Treg do not rely on glucose, this suggests the exciting possibility that Treg inhibit glucose metabolism as another mechanism of Teff suppression. It is conceivable that Treg inhibit glucose metabolism in Teff through the downregulation of Glut1 surface protein. Because there are not Glut1-specific surface antibodies available for sensitive flow cytometry, this could be tested with Glut1-myc mice. These mice express Glut1 protein with a tandem Myc epitope tag that is expressed under the endogenous promoter and therefore transporters and traffics glucose normally (Michalek *et al.*, 2011a). To test the hypothesis that Treg inhibit glucose metabolism as a means of suppression, Treg can be co-cultured with Glut1-myc Teff in a *in vitro* suppression assay and Glut1 surface expression can be tracked by flow cytometry to see if Glut1 gets downregulated upon Treg suppression.

Additionally, Treg suppression assays can be performed using Glut1 knockout or Glut1 transgenic Teff. Our data would suggest that Treg suppression would be less effective in Glut1 transgenic Teff in which Glut1 is overexpressed and glucose uptake is highly increased. This could then be taken into an inflammatory model, such as colitis, to test this hypothesis *in vivo*. In this setting, Glut1 transgenic Teff would be co-injected with wildtype Treg into Rag1^{-/-} mice to initiate colitis. If Treg are less able to suppress Glut1 transgenic Teff, we would predict the colitis disease progression to be more severe than with wildtype Teff. Similarly, we could examine Glut1-myc T cells *in vivo* to determine the time course of Glut1 up- or down-regulation during colitis disease progression with or without the presence of Treg. This could be done both with co-injection of Treg or alternatively, using a colitis model where colitis is initiated with Teff and then 21 days later, Treg are injected and are able to suppress colitis at this later time point (Chunder *et al.*, 2012). Both models would provide valuable information about the necessity of Glut1 to both disease progression and resistance or sensitivity to Treg suppressive abilities.

While this work focused on differences in glucose metabolism, there is little understanding of Treg lipid and glutamine utilization. In previous studies, our lab showed that Treg have high levels of lipid oxidation and are sensitive to etomoxir treatment (Michalek *et al.*, 2011a). However, etomoxir can have off-target effects and therefore a better approach would be to genetically delete a component of fatty acid

oxidation, such as CPT1 α , in T cells. An alternative strategy is to either overexpress CPT1 α or a CPT1 α shRNA lentiviral construct in primary T cells. Using these techniques, the requirement of Treg for lipid oxidation could be directly tested. It remains to be seen whether Treg primarily use lipids as a fuel or if they are simply able to use them but prefer glucose or another fuel. Additionally, our previous studies also suggest that lipids have a negative effect on Teff (Michalek *et al.*, 2011b). This could also be tested with the above strategies and examine if CPT1 α induction in Teff has a negative impact on effector proliferation or function. It is possible to imagine that the induction of lipid oxidation would be unfavorable for Teff, given that DCA or PDHK1 shRNA to drive glucose oxidation inhibits Th2 and Th17 differentiation and function.

In contrast to glucose and lipid metabolism, glutamine metabolism in the context of T cell differentiation is not well understood. However, there are a few studies looking at glutamine metabolism during T cell activation. Wang *et al.* showed that Myc-driven glutaminolysis is critical for T cell activation (Wang *et al.*, 2011) and Sinclair *et al.* demonstrated that T cell activation leads to the upregulation of a particular amino acid transporter subunit, Slc7a5, and was required for the activation of mTORC1 and the expression of Myc (Sinclair *et al.*, 2013). This suggests that glutamine and other amino acid metabolism is important for T cell metabolic reprogramming and so it will be important to examine in the context of T cell differentiation.

Given that some of the Treg suppression mechanisms involve amino acid utilization or starvation, it is likely that Teff and Treg have different glutamine or other amino acid requirements. Therefore, examining glutamine metabolism in the context of T cell differentiation will also be important to gain a more complete understanding of Teff and Treg fuel utilization. Ultimately, these differences in fuel utilization could be exploited in targeting specific T cell subsets. Glutamine starvation *in vitro* or inhibition using glutaminase inhibitors such as 6-diazo-5-oxo-L-norleucine (DON) will provide preliminary data on the requirement of differentiated T cells on glutamine metabolism (Willis *et al.*, 1977). While Wang *et al.* used Myc knockout T cells for this purpose; a more direct approach would be to use glutaminase (GLS) knockout T cells. Glutaminase is an enzyme that converts glutamine to glutamate and is an important part of glutamine utilization in the cell. Using GLS knockout T cells it will be possible to study the *in vivo* glutamine requirements of T cells in the context of colitis or other inflammatory disorder.

6.3 Regulation of glucose metabolism in T cell differentiation

Another important question that arises from this work is how the metabolic differences between the T cell subsets are regulated. In Th17, there is evidence that ROR γ t can directly interact with HIF1 α and drive a glycolytic program (Dang *et al.*, 2011). Dang *et al.* showed that HIF1 α activates ROR γ t via a complex with p300 and

regulates Th17-specific genes including IL-17. In our study, we examined the role of FoxP3 in Treg metabolism. Given that FoxP3 is the major transcriptional regulator in Treg, we hypothesized that FoxP3 may influence Treg metabolism, either directly or indirectly.

Several approaches were taken to examine this hypothesis. Knocking out FoxP3 in Treg is not useful because FoxP3⁻ T cells are no longer suppressive and thus no longer defined as Treg. Therefore, we generated a cell line where FoxP3 can be inducibly activated and metabolism could therefore be examined pre- and post-FoxP3 activation. We used a FoxP3-ER construct where FoxP3 is conjugated to a fragment of the estrogen receptor and remains in the cytoplasm until tamoxifen (*in vitro*: 4-hydroxytamoxifen) is added to translocate the FoxP3-ER to the nucleus where FoxP3 can initiate its transcriptional activity. The FoxP3-ER cell lines were generated using the FL5.12 pro-lymphocyte cell line because it does not normally express FoxP3 but is a lymphocyte precursor line that has been used to model T cell metabolism previously (Wofford *et al.*, 2008; Rathmell *et al.*, 2000). Using this model, the role of FoxP3 on metabolism was examined.

In our study, we examined the effect of FoxP3 on metabolism through both retroviral expression in primary CD4⁺CD25⁻ T cells or in the inducible FoxP3-ER lines. Through these studies we found that FoxP3 drives an oxidative metabolic program that promotes both glucose and lipid oxidation while suppressing glucose uptake and lactate

production. These metabolic changes may be due to both direct and indirect gene transcription and further work is needed to determine the mechanism of these metabolic changes. FoxP3 does have direct target genes that are metabolic in nature and further work will examine these direct target genes in greater detail by knockdown experiments. It is likely that in addition to direct metabolic target genes, FoxP3 drives the expression of other transcription factors that lead to metabolic changes.

For example, the transcription factor Foxo1 has recently been found to control Treg development and function. Mice that have Foxo1-deficient T cells have multiorgan lymphocyte infiltration (Kerdiles *et al.*, 2010). Additionally, Foxo1 knockout T cells differentiated into Th1 cells even in the presence of TGF β (Kerdiles *et al.*, 2010). In the context of skeletal muscle and adipocytes, Foxo1 plays a metabolic role, maintaining glucose homeostasis and promoting lipid oxidation during fasting conditions (Bastie *et al.*, 2005). This suggests that Foxo1 may be playing a metabolic role in Treg, either to promote Treg function through metabolic changes or as an additional role. Therefore, it would be interesting to examine the metabolic profile of Foxo1-null Treg to see if they have altered metabolism in addition to their lack of function.

Another important transcription factor that regulates oxidative metabolism is peroxisome proliferator-activated receptor gamma coactivator 1-alpha/beta (PGC1 α/β). This family of transcription factors is involved in the control of energy metabolic pathways, primarily mitochondrial pathways. Together with downstream transcription

factors including $ERR\alpha$ and the peroxisome proliferator-activated receptor (PPAR) family, PGC1 promotes electron transport, oxidative phosphorylation and fatty acid oxidation (reviewed in Finck *et al.*, 2006). Interestingly, PGC1 has been shown to directly associate with and activate Foxo1 in the context of liver metabolism (Puigserver *et al.*, 2003). FoxP3 may promote the expression of Foxo1, PGC1 or both as a mechanism to promote oxidative metabolism in Treg.

Another important signaling pathway in T cells is the Akt/PI3K/mTOR pathway. Previous studies have pointed towards a role of mTORC1/2 in T cell differentiation, although the role of mTOR in T cells appears to be complex. Delgoffe *et al.* showed that mTOR knockout T cells fail to become Teff even when differentiated *in vitro* with the appropriate cytokines and instead default to the Treg phenotype (Delgoffe *et al.*, 2009). In a later paper, Delgoffe *et al.* showed that T cells lacking mTORC1 activity preferentially differentiate into Th2 but not Th1/17, and alternatively T cells lacking mTORC2 activity can differentiate into Th1 and Th17 but not Th2 (Delgoffe *et al.*, 2011). Additional data from Zeng *et al.* shows that Treg can be generated from Raptor-deficient (mTORC1-deficient) T cells but they are not functional, however when mTORC2 is also deficient, the Treg phenotype is rescued (Zeng *et al.*, 2013). Therefore, the literature on mTOR signaling in T cell differentiation is both complex and controversial; however this pathway plays an important in Teff and Treg differentiation. The mTOR pathway affects a variety of signaling pathways, including metabolic pathways, and may be an

important contributor towards Teff and Treg metabolic profiles (Chi, 2012). It may be that fine-tuning of mTORC1/2 complexes promotes a particular metabolic program. Indeed, Zeng *et al.* demonstrated that Raptor-deficient Treg had reduced lipid and cholesterol synthesis (Zeng *et al.*, 2013). In our studies, we took a metabolic approach to examine the role of glucose metabolism in the T cell subsets and showed that Glut1 plays an important role in Teff, but not Treg differentiation and function. It is possible that the mTOR signaling pathway influences Glut1 and other metabolic proteins as a mechanism for the effect of mTOR on T cell differentiation.

In addition to further studies on the role of FoxP3 or other transcription factors on Treg metabolism, very little is known about the transcriptional programs underlying Th1 and Th2 metabolism. Therefore, a similar approach that was taken with FoxP3 should be taken with T-bet and GATA3 to examine the role of these important transcription factors on Th1 and Th2 metabolism. It may be that each transcription factor activates a unique set of metabolic genes, either directly or by influencing one of the above mentioned transcription factor or signaling pathway. Understanding the transcriptional programs underlying the T cell subset metabolic profiles is crucial to determine mechanisms of modulating T cell differentiation in the context of autoimmunity.

While many transcription factors including Myc, ERR α and HIF1 α are known to contribute to T cell metabolism, it is intriguing to consider the possibility that T cell

metabolism influences gene expression. There is now an appreciation for metabolic regulation of epigenetics. Epigenetics involves modifications to DNA and associated proteins, including acetylation, methylation and SUMOylation, that influences gene expression. DNA acetylation is initiated by histone acetyltransferases (HATs), which transfer an acetyl CoA group to the lysine residue of a DNA histone, typically promoting gene expression. Therefore, the availability of acetyl-CoA would be a determining factor in histone acetylation (reviewed in Lu *et al.*, 2012). Additionally, there is evidence that histones can also be O-linked N-acetylglucosamine (GlcNAc) modified using glucose as a starting product (Fujiki *et al.*, 2011). Therefore, the metabolic profile of each T cell subset may in turn contribute to epigenetic changes that affect gene transcription and T cell function. This could be tested by treating T cells with 2-deoxyglucose to inhibit glucose metabolism and examine the transcription of key genes including cytokines and transcription factors associated with each subset. Additionally, acetate can be directly added to cultured cells *in vitro* to provide acetylation substrate and see how this affects T cell gene expression (Wellen *et al.*, 2009). It is likely that signaling pathways in each T cell subset contributes to T cell metabolism, and the metabolic profile in turn affects T cell gene expression and both contribute to the overall T cell subset phenotype.

6.4 T cell metabolism and inflammatory disease

The objective of this work is to establish the role of glucose metabolism in T cell differentiation and ultimately, a metabolic strategy to target effector T cells in autoimmune and inflammatory disease. Currently, therapies for autoimmune disease involve broad immunosuppression using glucocorticoids or cytostatic agents (Brogan *et al.*, 2000). These therapies non-selectively inhibit all immune cells and therefore have a lot of side effects including immunodeficiency, hyperglycemia and others. More recently, biologic therapies have been developed with some success, mainly in the area of rheumatoid arthritis (RA). These include monoclonal antibodies that bind to cytokines or components of lymphocytes, mainly B cells. While the newer biologic therapies are more specific, they still cause immunosuppression and may lead to adverse side effects like infections and drug-induced SLE (Rosman *et al.*, 2013). Therefore, there is a need to find strategies to inhibit specific effector T cell subsets and promote Treg in the context of inflammatory and autoimmune disease.

Inflammatory disorders encompass a category of diseases with an excessive and inappropriate immune response against healthy body tissue and are widely prevalent in developed countries (El-Gabalawy *et al.*, 2010). In particular, inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease arise from inappropriate T cell responses against the gut microbiota and lead to chronic inflammation and intestinal damage (Zenewicz *et al.*, 2009). In the healthy gut, barrier functions of the colon

epithelial cells, as well as immunosuppression by the gut commensal bacteria, help to maintain homeostasis despite constant immune stimulation from food and bacterial antigens (Hooper *et al.*, 2001). IBD is therefore caused by both a disruption of the mucosal barrier, as well as an excessive Teff response or deficient Treg response causing intestinal inflammation. Indeed, Treg are extremely important to prevent inflammation, and when mice are depleted of Treg the major site of inflammation is in the intestinal tract (Powrie *et al.*, 1994).

Although there is a clear role of the Th1 cytokine IFN γ in IBD pathogenesis, recent data suggests an important role for Th17 in IBD (Powrie *et al.*, 1994). Th17-related cytokines are upregulated in patients with IBD and IL-23 has been found to play a significant inflammatory role (Yen *et al.*, 2006). Work from Izcue *et al.* suggests that IL-23 controls the frequency of Treg in the gut and in the presence of IL-23, there is a decrease in Treg and this promotes colitis (Izcue *et al.*, 2008). Therefore, therapeutic strategies to inhibit Th17 while preserving or increasing Treg will be important in the treatment of IBD.

In the present study, we show that Glut1 deficiency in Teff decreases T cell proliferation and function and therefore IBD severity. In contrast, Glut1 deficiency does not affect Treg, as Glut1 knockout Treg are still able to control Teff function and prevent IBD. Additionally, mice treated with the PDHK1 inhibitor DCA develop less severe IBD and this is associated with a decrease in IL-17 producing cells. Importantly, IFN γ

production was unaffected. This suggests that the inhibition of Th17 through DCA treatment is sufficient to inhibit IBD progression without affecting Th1. *In vitro*, DCA treatment increases Treg however this did not reach statistical significance in the IBD model. One reason for this is that the mice were injected with CD4⁺CD25⁻CD45RB^{hi} naïve effector T cells, which were depleted of natural Treg. Therefore, it will be important to examine Treg in IBD upon DCA treatment. This could be done by co-injecting low doses of Treg along with the naïve Teff cells, followed by DCA treatment, to see if DCA increases Treg in the context of IBD. In the setting of EAE, DCA did increase the Treg percentage *in vivo*. Overall, our studies suggest that the modulation of T cell metabolism may allow for the targeting of specific Teff subsets to modulate the immune response. DCA itself is an intriguing prospect, as it has been used in humans and was well tolerated in both children and adults during clinical trials for lactic acidosis (Stacpoole *et al.*, 1992). DCA is currently in clinical trials for glioblastoma and recurrent brain tumors. It will be important to focus future studies on how DCA affects the T cell metabolic profile. This can be done with metabolomics or C13-glucose labeling to look at metabolite changes. It will also be critical to examine the effect of DCA on other immune cell types, including CD8, B cells and antigen presenting cells. Finally, DCA treatment could be tested in other animal models of autoimmunity including allergic asthma, where both Th2 and Th17 cells play a major role.

IBD is an excellent model for examining Treg function, as disease spontaneously forms in the absence of Treg and alternatively can be rescued by the addition of Treg, either concurrently or after disease progression (Chunder *et al.*, 2012). However, an important consideration is the possibility that different tissues have distinct metabolic profiles that can contribute to T cell biology. As the GI tract is the site of food adsorption, it brings up the possibility that nutrient availability may affect the gut microbiota and/or immune cell function in the intestinal tract. Indeed, several recent studies have examined the relationship between nutritional status, gut microbiota and the immune response (reviewed in Kau *et al.*, 2011). One study found that short-chain fatty acids (SCFAs), which are provided by the gut bacteria, could promote the integrity of the epithelial barrier and also regulate the size of the Treg pool in the colon (Smith *et al.*, 2013). Mice that had been injected with T cells to induce IBD were treated with SCFAs and this was able to prevent IBD progression in a Treg-dependent manner.

The microbiota also affects the absorption of vitamins and minerals. Vitamin A (retinoic acid) deficiency can lead to a loss of Th17 cells and affects the balance between Th17 and Treg (Cha *et al.*, 2010). Further, iron deficiency protects mice against EAE and iron supplementation altered the composition of the gut microbiota and exacerbated Crohn's disease in a mouse model (Grant *et al.*, 2003; Werner *et al.*, 2011). These studies suggest that there is an important interaction between nutritional status, the gut microbiome and T cells. This is a significant area of study and could be expanded to

examine IBD and T cell dysregulation in the context of different nutrient diets. For example, putting mice on a ketogenic diet may prevent T cell glucose metabolism and thereby inhibit Teff and IBD progression.

There is also a link between obesity and the immune system. Obesity is associated with the expression of many inflammatory cytokines including IL-6, IL-17 and TNF α . There is even evidence for a unique Treg population found in visceral adipose tissue (VAT) of obese animals (Cipolletta *et al.*, 2012). This was associated with an increased PPAR γ expression in this Treg population, and the finding that VAT Treg contribute to the ability of pioglitazone to regulate insulin sensitivity. Additionally, the adipokine leptin functions as a T cell cytokine and has a variety of direct T cell effects (Procaccini *et al.*, 2012; Saucillo *et al.*, 2013). Overall, it remains to be seen whether changes in whole body nutritional status affect T cell function or differentiation but is an important area of investigation.

The finding that a unique Treg population exists in VAT brings up the interesting possibility that the T cell subsets have tissue-specific metabolic profiles that may affect their function. Differences in nutrient availability, or even oxygen status and hypoxia, may alter T cell signaling pathways and influence their functional properties. It will be important to examine the metabolic profile of T cells in different tissues to determine whether the metabolic profiles we have described are illustrative of all T cells of a particular population or if there is variability based on tissue distribution.

6.5 Concluding Remarks

The work presented here examines the metabolic phenotypes and regulation of effector and regulatory CD4 T cells and potential metabolic targets that could be used to treat autoimmune disease. While cellular metabolism has always been appreciated as a mechanism of energy production, it is often thought that metabolism is controlled by allosteric regulation and feedback product inhibition of metabolic enzymes. In contrast, the work presented here argues that the same signals that drive T cell activation and differentiation may set up a metabolic program that is appropriate and required for each specific cell type. Instead of upregulating glycolysis as a byproduct of glucose limitation, effector T cell metabolism is linked to differentiation and glucose metabolic pathways may be upregulated in anticipation of metabolic and functional needs. Similarly, the Treg-specific transcription factor FoxP3 drives an oxidative metabolic program that is required for optimal Treg suppressive function. Therefore, T cell metabolism is intimately linked to CD4 T cell survival, differentiation and function.

These data also suggest that targeting cell metabolism may provide a new opportunity for immune therapy. Each of the effector T cell subtypes and regulatory T cells play a unique role in the immune system, and with those roles have a distinct metabolic need to provide the proper fuels and biosynthetic precursors necessary for function. These metabolic differences may provide an opportunity to modulate the immune response by selectively targeting a particular cell type. This work describes

several strategies of metabolic inhibition, with Glut1 deficiency suppressing Teff but not Treg function, as well as PDHK inhibition as a mechanism to specifically inhibit Th17 and promote Treg. Future studies are needed to examine the immunometabolism of other cell types, as well as in the context of autoimmunity or inflammatory disease. Ultimately, a complete understanding of T cell metabolism and its regulation may lead to targeted immune therapies and improve the treatment of human autoimmune disease.

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Publications

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Awards and Honors

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